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Obesity and environmental pollutants: a reason for concern?

Mélanie Audrey Gomes Barbosa

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Obesity and environmental pollutants: a reason for concern?

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Resumo

A obesidade foi classificada pela Organização Mundial de Saúde no top 10 dos maiores riscos de saúde no mundo. Esta condição médica é um sério fator de risco para o desenvolvimento de diabetes de tipo 2, hipertensão, doenças cardiovasculares, esteatose hepática, como também de determinados cancros, podendo custar aos países cerca de mil milhões de euros anualmente, quer em tratamentos diretos ou indiretos. Para além de atingir proporções epidémicas por toda a humanidade, descobertas recentes apontam para uma associação entre a obesidade e os químicos ambientais, denominados "químicos obesogénicos", capazes de alterar o metabolismo lipídico e afetar o reino animal em toda a sua extensão. Com o objetivo de elucidar o modo de ação destes compostos e avaliar a capacidade de misturas ambientais (efluentes de entrada de Estações de Tratamento de Águas Residuais) promoverem efeitos obesogénicos, combinamos um ensaio *in vivo* usando a espécie-modelo peixe-zebra (*Danio rerio*) e avaliações de alterações na expressão de genes essenciais para a lipogénese/adipogénese e lipólise, juntamente com um ensaio de transativação *in vitro*, focado na interação dos compostos com os recetores nucleares retinoid X receptor α (RXRα), peroxisome proliferator-activated receptor γ (PPARγ) e heterodímero RXRα:PPARγ do ser humano e do peixe-zebra. As larvas foram alimentadas com uma dieta rica em lípidos durante todo o ensaio e foram expostas, desde a sua saída do ovo até ao dia 18 pós-fertilização, aos seguintes tratamentos: Controlo, controlo solvente (DMSO), 100 ng/L e 200 ng/L Sn do composto obesogénico modelo, tributilestanho (TBT), 1.25% e 2.5% de efluente de entrada de ETAR. Um acréscimo significativo na acumulação lipídica foi detetado em todos os tratamentos com TBT e efluente de ETAR, com a exceção do efluente a 1.25%, enquanto a expressão de genes aumentou significativamente para todos os genes lipogénicos/adipogénicos em ambas as concentrações de efluente de ETAR. Os tratamentos com TBT não exibiam alterações na expressão génica, exceto em FASn na concentração de 100 ng/L Sn. Os resultados da transativação indicaram uma ativação significativa do heterodímero humano e repressão do heterodímero de peixe-zebra no tratamento com 250 nM de TBT, enquanto o efluente a 10% ativou apenas o heterodímero humano. Em geral, o nosso estudo mostra a capacidade de amostras ambientais e compostos obesogénicos causarem a disrupção da homeostasia lipídica através da interação com os recetores RXRα e/ou PPARγ, promovendo a obesidade enquanto alteram a expressão de genes e enzimas "chave" das vias reguladas por estes recetores nucleares.

Palavras-chave: obesidade, químicos obesogénicos, disruptores endócrinos, receptor nuclear, adipogénese, lipogénese.

Abstract

Obesity has been ranked in the top 10 health risks in the world by the World Health Organization. This medical condition is a serious risk factor for the development of type-2 diabetes, hypertension, cardiovascular disease, hepatic steatosis, as well as some cancers, and can cost countries billions of euros annually in direct or indirect care. Besides reaching epidemic proportions in human beings all around the world, recent findings point to an association between obesity and environmental chemicals, named “obesogens”, able to alter lipid metabolism and thoroughly affect the animal Kingdom. Aiming to elucidate the mode of obesogen action and assess the ability of environmental mixtures (WWTP influent) to promote obesogenic effects, we have combined an *in vivo* assay using the model species zebrafish (*Danio rerio*), and evaluations of the altered expression of key genes involved in lipogenesis/adipogenesis and lipolysis, with an *in vitro* transactivation assay with human and zebrafish nuclear receptors retinoid X receptor α (RXRα), peroxisome proliferator-activated receptor γ (PPARγ) and the RXRα:PPARγ heterodimer. The zebrafish larvae were fed with a high fat diet throughout the experiment and exposed from hatching to the 18th day postfertilization to the following treatments: Control, Solvent control (DMSO), 100 ng/L and 200 ng/L Sn of model obesogen tributyltin (TBT), 1.25% and 2.5% of WWTP influent. A significant increase in lipid accumulation was detected in all exposure treatments except for 1.25% influent, whereas gene expression was enhanced for all lipogenic/adipogenic genes in both WWTP treatments. TBT treatments did not exhibit changes in gene expression, with the exception of FASn at 100 ng/L Sn TBT. Transactivation results indicated a significant activation of the human heterodimer and repression of the zebrafish heterodimer in the treatment with 250 nM TBT, while WWTP influent at 10% activated the human heterodimer. In general, our study demonstrates the ability of environmental samples and a model obesogen to cause the disruption of lipid homeostasis through interaction with the RXR and/or PPARγ receptors, therefore promoting obesity whilst modulating the expression of key genes from downstream pathways.

Keywords: obesity, obesogens, endocrine disruptors, nuclear receptor, adipogenesis, lipogenesis.

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ABBREVIATION	DESCRIPTION
ACC	Acetyl-CoA carboxylase
ACOX	Acyl-CoA oxidase
BPA	Bisphenol A
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer-binding protein
CPT1	Carnitine palmitoyltransferase I
CRH	Corticotropin-releasing hormone
DBD	DNA binding domain
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Diethylhexylphthalate
DES	Diethylstilbestrol
DMSO	Dimethylsulfoxide
dpf	Days postfertilization
EDC	Endocrine disrupting chemical
ER	Estrogen Receptor
FABP4	Fatty acid binding protein 4
FAS	Fatty acid synthase
FXR	Farnesoid X receptor
GPAT	Glycerol-3-phosphate acyltransferase
GR	Glucocorticoid receptor
HNF4A	Hepatocyte nuclear factor 4 alpha
HPA	Hypothalamic-pituitary-adrenal
HR	Hinge region
IGF	Insulin-like growth factor
LBD	Ligand binding domain
LSD	Least Significant Difference
LXR	Liver X Receptor
MEHP	Monoethyl-hexyl-phthalate
METBP	Mono-(2-ethylhexyl) tetrabromophthalate
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSC	Mesenchymal stem cell

NR	Nuclear receptor
OCP	Organochlorine pesticide
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PFC	Perfluoroalkyl compound
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
POP	Persistent organic pollutant
PPAR	Peroxisome proliferator-activated receptor
PTM	Post-translational modification
PXR	Pregnane X Receptor
qPCR	Quantitative real-time Polymerase Chain Reaction
RXR	Retinoid X Receptor
SEM	Standard error of the mean
SREBP	Sterol regulatory element-binding protein
TBBPA	Tetrabromobisphenol-a
TBBPA-S	Tetrabromobisphenol-a sulfate
TBT	Tributyltin
TCBPA	Tetrachlorobisphenol-A
TPhT	Triphenyltin
TZD	Thiazolidinedione
WAT	White Adipose Tissue
WWTP	Wastewater treatment plant
HSD	Hydroxysteroid dehydrogenase

1. Introduction

1.1. The obesity epidemic

Obesity, also known as adiposity, is characterized by an abnormal accumulation of fat and increased amount and size of fat cells in the body that may result in the impairment of health, not only in adults but also in adolescents and young children (Chamorro-Garcia & Blumberg, 2014; Grün & Blumberg, 2009; Landgraf *et al.,* 2017; Newbold *et al.,* 2007). Its prevalence, as well as the ones of its associated diseases, is reaching epidemic proportions all around the globe, affecting primarily industrialized and western countries, such as the United States (Grün & Blumberg, 2009; Minchin & Rawls, 2011; Newbold *et al.,* 2007; Santos *et al.*, 2012). Consequently, obesity has been ranked as one of the top 10 health risks in the world, by the World Health Organization, as it is estimated that the number of people with excessive weight is currently larger than the number of undernourished people in the world (Newbold *et al.,* 2007). This medical condition can cost countries billions of dollars per annum in either direct or indirect care (estimates for the USA: $147 billion in 2008, equivalent to around 128.8 billion €) (Spencer & Tilbrook, 2011).

Obesity is a serious risk factor for the development of metabolic syndrome-associated disorders (dyslipidemias), type-2 diabetes, hypertension, cardiovascular disease, nonalcoholic fatty liver disease (also known as hepatic steatosis), asthma and pulmonary problems, osteoarthritis, gall bladder and kidney disease premature mortality and some cancers as well (Grün & Blumberg, 2009; Landgraf *et al.,* 2017; Minchin & Rawls, 2011; Newbold *et al.,* 2007). Furthermore, weight disruption has been linked to psychological problems, such as poor self-esteem, depression and social discrimination (Newbold *et al.,* 2007) and also neurological disorders such as schizophrenia and bipolar disorder, besides being a side effect of the use of pharmaceutical treatments designed to treat these disorders (atypical antipsychotics, tricyclic antidepressants and selective serotonin reuptake inhibitor antidepressants) (Grün & Blumberg, 2009).

Even though this medical condition develops primarily from a chronic positive energy balance, which means that energy intake exceeds energy expenditure (Grün & Blumberg, 2009; Minchin & Rawls, 2011), its exact causes are still uncertain (Newbold *et al.,* 2007; Santos *et al.* 2012). In fact, there are several factors regulating fat accumulation and distribution, as well as the mobilization of lipids from adipose tissue, which do not result only from overeating and exercise (Grün & Blumberg, 2009), as this condition is thought to be caused by a complex interaction between genetic, behavioral and environmental factors (Newbold *et al.,* 2007; Spencer & Tilbrook, 2011). Hence, the existence of single nucleotide

polymorphisms in some genes and genetic heritage, the exposure to viruses, the conditions of the intestinal microbiome, psychological stress and chronic reductions of sleep, can also influence obesity (Bašić *et al.,* 2012; Chamorro-Garcia & Blumberg, 2014; Grün & Blumberg, 2009; Santos *et al.,* 2012; Spencer & Tilbrook, 2011). In fact, although body fat distribution is heritable in mammals, which suggests that different adipose depots have their own unique developmental gene expression signature, these also respond differently to nutritional status (Birsoy *et al.,* 2013).

Furthermore, recent findings seem to point out the existence of a new factor influencing obesity: chemical exposure. These chemicals, known as "obesogens", which can be natural or xenobiotic (Janesick & Blumberg, 2011), are able to alter lipid homeostasis directly through stimulation of adipogenesis and fat accumulation, or indirectly through regulation of appetite and satiety (Chamorro-Garcia & Blumberg, 2014; Grün & Blumberg, 2009; Janesick & Blumberg, 2011; Newbold *et al.,* 2007; Santos *et al.,* 2012). They can possibly explain the rapid increase in obesity rates that coincided with the augmented release of industrial chemicals in the past four decades (Newbold *et al.,* 2007; Santos *et al.,* 2012).

1.2. Lipid homeostasis

Lipid homeostasis is crucial to ensure the energy balance of organisms but also for the proper functioning of cells (Santos *et al.,* 2012). Owing to their variety in chemical properties, lipids are able to participate in a diversity of functions within the cell and in the whole organism. They play essential roles in the energy storage (triacylglycerol and cholesterol ester), are part of the structure of biological membranes (phospholipids and sterols) and can be used as intracellular messengers, enzyme cofactors, electron carriers, light-absorbing pigments, hydrophobic anchors for proteins, hormones, "chaperones" to help membrane proteins fold and also as precursors for biosynthesis (Baeza-Jiménez *et al.,* 2014; Birsoy *et al.,* 2013).

All animals have developed mechanisms to maintain energy reserves and store fat in order to survive periods where food is scarce (Birsoy *et al.,* 2013; Santos *et al.,* 2012).

Several different body organs contribute to the maintenance of the energy homeostasis. The central nervous system plays an essential role as a coordinator of all processes involved in the homeostasis, as it acts as a lipid and hormone sensor and integrates afferent information from different organs, transforming it into signals that will ensure the necessary adjustments, for example, through a change in feeding behavior or energy expenditure (Lyssimachou *et al.,* 2015; Santos *et al.,* 2012). The muscle is the main organ involved in energy expenditure and lipid oxidation (Lyssimachou *et al.,* 2015). The liver plays a central role in glucose and fatty acids synthesis, metabolism and distribution. Due to the liver's

short capacity to accumulate fat, it is a good indicator of diseases such as the non-alcoholic fatty liver, hepatocyte apoptosis and hepatic steatosis (Lyssimachou *et al.,* 2015). Moreover, most obesogenic compounds have the liver as the primary target organ (Maradonna *et al*., 2015). In the mammalian liver, fatty acid synthesis begins in the cytosol with the conversion of acetyl-CoA into malonyl-CoA by acetyl-CoA carboxylase (ACC), followed by the synthesis of fatty acids catalyzed by the fatty acid synthase complex (FAS), which converts malonyl-CoA into a palmitate (Guan *et al.,* 2016; Kersten *et al.,* 2001, Santos *et al.,* 2012). Finally, triglycerides are formed from the esterification of the palmitate molecule (Guan *et al.,* 2016; Kersten *et al.,* 2001).

Lipogenesis includes the synthesis of fatty acids and subsequent triglyceride synthesis and can occur in both the liver and the adipose tissue (Kersten, 2001). *De novo* lipogenesis is the process through which carbohydrates and protein from the diet are transformed into fat and occurs due to the inexistence of a mechanism for direct storage of a long-term supply of these nutrients (Santos *et al.,* 2012). Excess energy is stored in the form of triglycerides in the adipose tissue, until it is converted to fatty acids and released into the blood stream when energy sources are low (Lyssimachou *et al.,* 2015; Moseti *et al.,* 2016). After a prolonged positive energy balance, it is possible for triglycerides to be stored in the muscle and liver as well, which can potentially lead to metabolic disorders (Lyssimachou *et al.,* 2015). Moreover, while fat accumulation in the subcutaneous adipose tissue is considered an adaptive process, exceeding its storage capacity diverts the excess of fatty acids towards the accumulation in the visceral cavity, which, on the contrary, is associated to adverse health consequences (Chamorro-Garcia & Blumberg, 2014).

In Metazoans, lipid accumulation occurs in specific cell types, in contrast to vertebrates that own a whole tissue dedicated to lipid storage: the adipose tissue (Birsoy *et al.,* 2013). Around 50 to 60% of the mammalian adipose tissue is composed of adipocytes, with the remaining cells types being endothelial cells, pericytes, fibroblasts, leukocytes, neurons and preadipocytes (Birsoy *et al.,* 2013). This tissue functions as an active endocrine and immune organ, secreting pro- and anti-inflammatory adipokines (e.g. leptin, resistin and adiponectin) in abundance that regulate metabolic processes by acting on the central nervous system and peripheral tissues involved in adiposity, glucose homeostasis, food intake and lipid metabolism, among other processes (Bašić *et al.,* 2012; Birsoy *et al.,* 2013; Santos *et al.,* 2012).

The process by which precursor stem cells (preadipocytes) differentiate into lipid-loaded mature adipocytes, adipogenesis, is controlled by a complex gene expression program (Moseti *et al.,* 2016; Kersten, 2001) and comprises two stages: the commitment of stem

cells into adipocyte precursors and the terminal differentiation of preadipocytes into mature fat cells (Birsoy *et al.,* 2013). *In vitro* studies with embryonic stem cells, mesenchymal stem cells (MSC) and 3T3L1 fibroblasts (preadipocytes) are the most common for the identification of key regulators of this process: among others, CCAAT/enhancer-binding protein (C/EBP) β, C/EBPδ and sterol regulatory element-binding protein (SREBP-1) as early regulators of adipogenesis, peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα for the final adipocyte maturation where they will modulate the expression of other adipocyte-specific genes (Birsoy *et al.,* 2013; Moseti *et al.,* 2016; Santos *et al.,* 2012). The fatty acid binding protein 4 (FABP4), adiponectin, and fatty acid synthase (FAS) are needed for the maturation of adipocytes (Moseti *et al.,* 2016).

The distribution of the adipose tissue varies across vertebrates. In fish, amphibian and reptiles, the tissue is located mainly in intra-abdominal regions and rarely in the subcutaneous area, while in mammals it is distributed throughout the body (Birsoy *et al.,* 2013). In lower exothermic animals, the tissue is usually small since the lipid storage is mainly performed by the liver, which also secretes adiponectin and leptin, the mammalian orthologues of the adipokines (Birsoy *et al.,* 2013).

Other important factors for the maintenance of normal adipose tissue and its functions are androgens and estrogens (Grün & Blumberg, 2007). Sex hormones, alongside with the growth hormone, counteract the effects of cortisol and insulin by mobilizing the fat instead of accumulating it (Grün & Blumberg, 2007; Grün & Blumberg, 2009), and are required to regulate adipocyte hypertrophy and hyperplasia (Grün & Blumberg, 2009). Furthermore, different levels and ratios of sex steroids are associated with variations in adipose mass volume and distribution, as women tend to have a greater body weight, as well as a greater number and size of adipocytes, distributed mostly subcutaneously around puberty, followed by distribution in the abdominal area during menopause, which is provoked by the lower estrogen level and changes in estrogen-androgen ratio (Birsoy *et al.,* 2013; Grün & Blumberg, 2007; Yoon, 2009). The lower levels of estrogen in both ovariectomized animals and post-menopausal women are also associated, besides the increase in body weight and adipose mass, to increased food intake, since when given estrogen these symptoms are not displayed (Yoon, 2009).

Aside from steroid hormones, many other hormone systems are involved in weight control (e.g. catecholamines, thyroid hormones, insulin, growth hormone and leptin) and are also targeted by environmental chemicals (Bašić *et al.,* 2012; Santos *et al.,* 2012). Moreover, the sympathetic nervous system contributes to the control of body weight and fat

accumulation through the production of noradrenaline, dopamine and adrenaline (Santos *et al.,* 2012).

Hormones can either have an inhibiting effect (growth hormone and leptin) of stimulating effect (insulin) on lipogenesis (Kersten, 2001). The most well-known hormonal factor regulating lipogenesis is insulin, which increases the recruitment of glucose transporters to the plasma membrane thereby increasing the uptake of this nutrient, but also activates and stimulates the expression of lipogenic and glycolytic enzymes, thus stimulating lipogenesis (Kersten, 2001). The growth hormone promotes fat loss in the adipose tissue coupled with a gain of muscle mass, due to down-regulation of the expression of lipid synthesis genes (such as fatty acid synthase) and thus, reduced lipogenesis, either directly or through the decrease in insulin sensitivity (Kersten, 2001).

Leptin is an important adipokine produced by mature adipocytes, as its expression highly correlated with the amount of fat mass (degree of adiposity) (Birsoy *et al.,* 2013; Kersten, 2001). As the levels of leptin increase, it sends an afferent signal to the hypothalamus to decrease food intake, increase energy expenditure and inhibit lipogenesis through down-regulation of the expression of genes involved in fatty acid and triglyceride synthesis, in order to maintain the homeostasis (Birsoy *et al.,* 2013; Kersten, 2001). In fish, leptin is also expressed in the liver, biliary system and intestine (Birsoy *et al.,* 2013).

Insulin, glucose and leptin all seem to interact either positively (insulin and glucose) or negatively (leptin) with the Sterol Regulatory Element Binding Protein-1 (SREBP1), an important transcription factor that stimulates lipogenesis when activated (Kersten, 2001). This transcription factor mediates most of the effects of nutrients and hormones on the expression of lipogenic genes in the liver, while the same occurs through PPARγ in the adipose tissue (Kersten, 2001).

Diets with high carbohydrate content stimulate lipogenesis in both liver and adipose tissue, leading to elevated postprandial plasma triglyceride levels, while polyunsaturated fatty acids and fasting decrease lipogenesis, respectively, by suppressing gene expression in the liver (for example, of fatty acid synthase) and increasing the rate of lipolysis that will cause loss of triglycerides from the adipose tissue and increase in plasma-free fatty acids (Kersten, 2001).

Another factor of impact on the pathophysiology of obesity is psychological stress (Lyssimachou *et al.,* 2015; Spencer & Tilbrook, 2011). Stressful events cause a significantly faster weight gain that is associated with a dysfunctional regulation of the hypothalamic-pituitary-adrenal (HPA) axis due to higher basal cortisol levels (Grün & Blumberg, 2009; Lyssimachou *et al.,* 2015; Spencer & Tilbrook, 2011). The HPA axis is the key endocrine

axis that mediates the body's response to stress. In the presence of a stressor, cells of the hypothalamus are activated and stimulate the release of corticotropin-releasing hormone (CRH) and arginine vasopressin into the hypothalamohypophysial portal blood vessel system, thus releasing adrenocorticotropic hormone from the anterior pituitary into the systemic circulation that will stimulate glucocorticoid (cortisol in humans, corticosterone in rodents) synthesis at the adrenal cortex. Right after an acutely stressful event, the appetite will be suppressed at the brain level (through CRH) and be followed by an increase in appetite and feeding in the subsequent hours to days, which is tied to a peak in glucocorticoid release (Spencer & Tilbrook, 2011). It seems that this phenomenon is an adaptive solution to replace the loss of energy after an acute episode of stress, but also to prepare the body for a possible future stressful event, which raises concern if the stressor continues for too long or if there are multiple consecutive stressors, as the chronic increase in glucocorticoid levels can lead to a chronic stimulation of appetite (especially for high-energy foods) and as a result, obesity (Grün & Blumberg, 2009; Spencer & Tilbrook, 2011).

Glucocorticoids also play a role in adipocyte differentiation and in lipid homeostasis, as they enhance lipolysis (through activation of hormone-sensitive lipase) and promote fat storage (by enhancing adipose lipoprotein lipase activity), besides enhancing the appetite-stimulatory effects of ghrelin and reducing the brain's sensitivity to insulin and leptin, thus decreasing their ability to inhibit feeding-stimulatory pathways (Grün & Blumberg, 2009; Spencer & Tilbrook, 2011).

To sum up, both adipogenesis and lipid synthesis and accumulation involve complex transcriptional cascades, with cross-talks between many different signaling pathways, in which transcription factors are responsible for the expression of key enzymes involved in these processes (Moseti *et al.,* 2016; Santos *et al.,* 2012).

With so many factors influencing this disease, such as the hormonal regulation, regulation of glucose levels and the number, size and metabolic activity of adipocytes (Grün & Blumberg, 2009; Santos *et al.,* 2012), it is understandable that drugs have been developed to act at different levels: to decrease appetite and food intake, to increase energy expenditure and to regulate the metabolism and nutrient partitioning (Yoon, 2009).

1.3. The peroxisome proliferator- activated receptors

Peroxisome proliferator-activated receptors (PPARs), such as PPARγ, PPARα and PPARβ/δ are members of the ligand-activated transcription factors from the nuclear receptor (NR) superfamily and have been intensively investigated in mammalians (Santos *et al.,* 2012; Yoon, 2009). These receptors play essential roles in adipocyte differentiation and in the maintenance of lipid homeostasis, by forming an obligate heterodimer with the

retinoid X receptors (RXRs) and acting on DNA response elements in order to change the transcription of target genes (Santos *et al.,* 2012).

In the absence of a ligand, co-repressors are bound to nuclear receptors and chromatin is condensed, thus not allowing significant transcription of target genes (Janesick & Blumberg, 2011). In contrast, the binding of a ligand causes a conformational change in the receptor that will recruit co-activators and lead to the release of co-repressors, as well as the decondensation of the chromatin, to induce transcriptional activation (Janesick & Blumberg, 2011; Yoon, 2009). Interestingly, nuclear receptors can also be activated or unrepressed through post-translational modifications (PTMs) that cause active release of co-repressors in the absence of the NR ligands (Janesick & Blumberg, 2011).

Mammals have the three PPAR subtypes aforementioned, while teleost fish (e.g. zebrafish) may have up to five different PPAR genes (Santos *et al.,* 2012). Each subtype has different ligand specificity, tissue distributions and biological functions (Santos *et al.,* 2012; Yoon, 2009). Yet, as their natural ligands in mammals are fatty acids and lipid-derived substrates (Kersten, 2001; Santos *et al.,* 2012), they act as lipid sensors and integrate the control of energy homeostasis, lipid and glucose metabolism (Santos *et al.,* 2012).

The PPARγ is possibly the most important nuclear receptor involved in lipid homeostasis and adipogenesis. It is mostly expressed in adipocytes and is required in a variety of physiological processes, for example, adipogenic differentiation, inflammation and glucose metabolism (Moseti *et al.,* 2016), since it directly induces many genes that regulate the adipogenic cascade and lipid uptake, synthesis and storage (Birsoy *et al.,* 2013). In fact, this nuclear receptor is widely known as the “Master regulator of adipogenesis” (Birsoy *et al.,* 2013; Janesick & Blumberg, 2011; Lyssimachou *et al.,* 2015; Santos *et al.,* 2012), as it is massively induced during adipocyte differentiation (Moseti *et al.,* 2016) and amongst the several transcription factors involved in adipocyte maturation, only PPARγ is truly necessary for adipogenesis (Santos *et al.,* 2012).

Even though PPARγ activation is a necessary condition for the differentiation of preadipocytes into adipocytes, simply testing the activation of this receptor by a chemical was shown not to be enough to predict the obesogenic potential of the latter (Janesick & Blumberg, 2011), as other mechanisms of action are equally important for this purpose (Pereira-Fernandes *et al.,* 2013).

Several compounds have been shown to be agonists of this nuclear receptor in reporter gene assays, yet have not been linked to obesity. Moreover, some compounds might activate the receptor in some types of cells but not in others, as the recruitment of coregulators to PPAR target genes is variable (Janesick & Blumberg, 2011). A particularly

contradictory example is mycophenolic acid, a PPARγ-agonist in 3T3-L1 preadipocytes, that was shown to inhibit the adipogenesis in the same cells (Ubakata *et al.,* 2007). In 3T3-L1 cells, it was also shown that this NR can function as an unliganded receptor or that it might be activated by endogenous ligands (Walkey & Spiegelman, 2008).

The PPARγ:RXR heterodimer increases the expression of genes that exert different functions in distinct body organs (Santos *et al.,* 2012):

- Adipose tissue: energy expenditure, fatty acid storage, glucose uptake, adipocyte differentiation and increase or decrease of adipokines.
- Muscle: glucose uptake and glucose oxidation.
- Liver: energy expenditure, fatty acid uptake and gluconeogenesis.
- Macrophage: cholesterol efflux and lipid uptake.
- Pancreas: β-cell function.

Besides fatty acids, prostaglandins have also been suggested as natural ligands of PPARγ (Birsoy *et al.,* 2013).

The PPARγ is known to be sensitive to environmental chemicals (Janesick & Blumberg, 2011; Lyssimachou *et al.,* 2015), as its ligand-binding pocket is large enough to accept a variety of chemical structures (Janesick & Blumberg, 2011). Consequently, the existence of chemicals, such as endocrine disrupting chemicals (EDCs), that are able to interfere with these receptors pose a concern, as they might lead to the disruption of the homeostasis due to inappropriate regulation of the pathways (Santos *et al.,* 2012). Moreover, the obligatory PPARγ:RXR heterodimer is permissive, which means that it is possible to target either half of the heterodimer, as the signaling can be mediated by ligand activation of either half (Grün & Blumberg, 2009).

Another reason for concern is that, while PPARs are present in vertebrates and several invertebrate groups, RXR is common within metazoans and consequently, the disruption of these pathways through RXR might have a wider taxonomic scope than expected (Santos *et al.,* 2012; Lyssimachou *et al.,* 2015).

PPARα plays a central role in lipid and lipoprotein metabolism and regulates energy balance by promoting fat catabolism, decreasing dyslipidemia associated with metabolic syndrome (Yoon, 2009). It also promotes ketone body synthesis and glucose sparing (Santos *et al.,* 2012). Its expression is particularly high in tissues like the liver, heart, muscle, brown adipose tissue, skeletal muscle, kidney and intestinal mucosa, where the levels of mitochondrial and peroxisomal fatty acid catabolism are greater (Yoon, 2009). Natural ligands for this receptor are fatty acids and derivatives, yet it can also be activated by

synthetic compounds, such as, nonsteroidal anti-inflammatory drugs, carbaprotacyclin, pirinixic acid, phthalate ester plasticizers and hypolipidemic drug fibrates used to decrease the levels of circulating triglycerides and increase high-density lipoprotein cholesterol levels (Yoon, 2009).

PPARα suppresses the expression of acetyl-CoA carboxylase (ACC), which is the enzyme responsible for *de novo* lipogenesis and thus the reduction of its activity decreases the levels of intracellular fatty acids available for triglyceride synthesis. Moreover, inhibitors of ACC also enhance the oxidation of fatty acids, increasing energy expenditure in experimental animal and cultured cells (Yoon, 2009). Among other target genes of PPARα, fatty acid synthase (FAS) gene expression is also decreased, while the gene expression of Acyl-CoA oxidase (ACOX), an enzyme involved in peroxisomal β-oxidation, is increased by PPARα activation (Grün & Blumberg, 2009; Yoon, 2009).

To further complicate the mechanisms underlying obesity, even though both estrogens and PPARα exert anti-obesogenic effects, it appears that PPARα ligands only function as efficient controllers of obesity when the levels of estrogen are low (e.g. in men and postmenopausal women). There is a bidirectional signal cross-talk regulation between PPAR and Estrogen Receptor (ER), as they compete not only directly for DNA binding in the response elements, due to sequences that can be recognized by both receptors, but also for coactivators, availability of corepressors and other mechanisms. Consequently, PPARα ligands, such as fenofibrate, may be ineffective in treating lipid disorders in premenopausal women, since PPARα activity is inhibited in the presence of estrogen (Yoon, 2009).

Finally, PPARβ does not have a specific tissue distribution but also regulates fatty acid oxidation in the tissues where PPARα is either absent of less expressed (Santos *et al.,* 2012).

Regardless, developing anti-obesity therapies that promote weight loss and increase fat oxidation, for example, by targeting the PPARα, seems to be a good alternative strategy to the long-term use of drugs, such as sibutramine and orlistat, which have unwanted side effects (Yoon, 2009).

The factors influencing lipogenesis and/or adipogenesis are summarized in Figure 1.

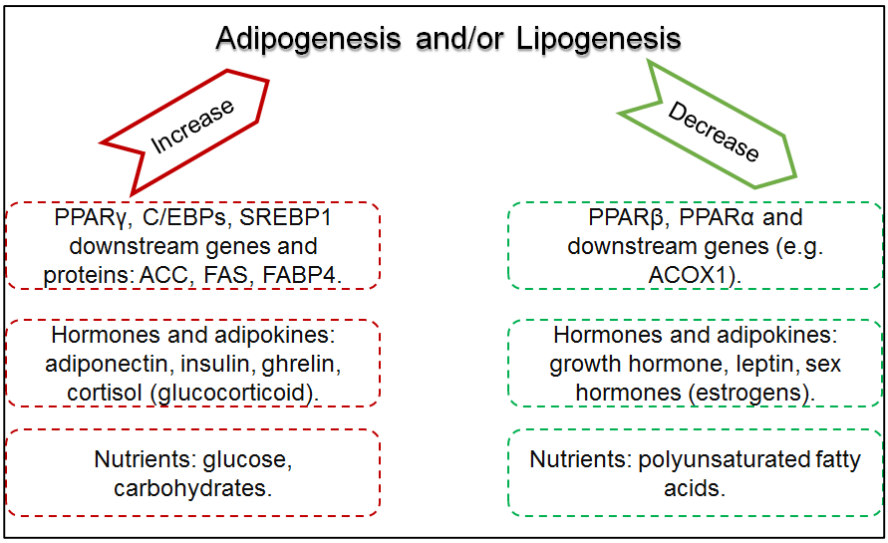

Figure 1. Scheme listing some of the many factors influencing obesity.

1.4. Identified obesogens and possible modes of action

Obesogens are a class of EDCs capable of interfering with the lipid metabolic pathways (Lysshimachou *et al.,* 2015). The disruption of the lipid homeostasis by EDCs is an emerging new field, as there is increasing evidence of their effects not only in humans but also animals such as pets, feral rodents and laboratory animals, living in proximity to human populations, that have also suffered weight gain (Chamorro-Garcia & Blumberg, 2014; Grün & Blumberg, 2009; Lyssimachou *et al.,* 2015; Newbold *et al.,* 2007). EDCs are known to mimic or block the role of natural hormones and interfere with nuclear receptors, dysregulating the metabolic pathways and thus, leading to changes in patterns of gene expression (Grün & Blumberg, 2007; Lyssimachou *et al.,* 2015; Newbold *et al.,* 2007). Some of these chemicals were initially characterized as “androgenic” or “estrogenic”, yet later have been found to also interact with nuclear receptors involved in lipid homeostasis (Lyssimachou *et al.,* 2015).

Exposure to chemicals *in utero* and during early development (critical periods of differentiation), can alter developmental programming leading to obesity that might not manifest until later in life (Bašić *et al.,* 2012; Grün & Blumberg, 2007; Newbold *et al.,* 2007; Santos *et al.,* 2012). Effects of this exposure can sometimes be transgenerational. Mesenchymal stem cells are pluripotent cells that can originate a variety of lineages (adipogenic, osteogenic and cartilagenic). Thus, if they are under stimuli that bias their fate towards the adipocyte lineage, it is possible that the animals are predisposed to possess more fat cells than they normally would (Chamorro-Garcia & Blumberg, 2014).

In regard to general environmental estrogens (e.g. diethylstilbestrol, bisphenol A and dichlorodiphenyltrichloroethane, DDT), these seem to promote anti-adipogenic effects in

adults, yet some of these compounds can still interact with non-estrogenic signaling pathways, resulting in adipogenic effects (Chamorro-Garcia & Blumberg, 2014; Grün & Blumberg, 2007). On the other hand, if exposure to estrogens occurs in the prenatal or early perinatal period, the effect is expected to be pro-adipogenic (Grün & Blumberg, 2007). Phytoestrogens, natural compounds found in soy products, such as genistein and daidzein, mimic estrogen action on adipogenesis and lipogenesis (Grün & Blumberg, 2007).

One possible mode of obesogen action is by targeting the PPARγ at a transcriptional level, through the modification of chromatin structure, aiding the expression of PPARγ during adipogenesis (Janesick & Blumberg, 2011). With regard to post-translational modifications (PTMs), mentioned in the previous subchapter, PPARγ is known to be phosphorylated, SUMOylated and ubiquitinated (van Beekum *et al.,* 2009). It has been suggested that targeting these PTMs could lead to an obesogenic potential if they stabilize the PPARγ protein and increase the transcription of adipogenic genes or regulate the receptor's interactions with the transcriptional machinery (Janesick & Blumberg, 2011).

It has also been suggested that obesogens could induce epigenetic modifications (e.g. altering the methylation patterns of promotor regions of genes or leading to histone modifications) in genes that are perhaps involved in the etiology of obesity (Bašić *et al.,* 2012; Chamorro-Garcia & Blumberg, 2014; Newbold *et al.,* 2007). Moreover, these epigenetic modifications might lead to transgenerational changes in gene expression (Chamorro-Garcia & Blumberg, 2014).

Genistein is a phytoestrogen reported to decrease obesity occurrence (Grün & Blumberg, 2007; Newbold *et al.,* 2007) by reversing the increase in fat accumulation in a gender specific and dose-dependent manner (Grün & Blumberg, 2007). It has been shown to increase the methylation of the promotor region of the A^{vy} allele, a gene associated with obesity, subsequently leading to its silencing in genetically altered mice that develop obesity and thus reverting its phenotype to a normal instead of an obese one (Newbold *et al.,* 2007). Therefore, the possibility that chemicals might have a similar mode of action, enhancing obesity, should be investigated.

So far, a few chemicals with obesogenic potential have already been identified: organotins (tributyltin, TBT, and triphenyltin, TPhT), thiazolidinediones (TZDs such as rosiglitazone and pioglitazone), phthalates (diethylhexylphthalate, DEHP, and mono-(2-ethylhexyl) tetrabromophthalate, METBP), bisphenol A (BPA) and its derivatives and flame retardants such as polybrominated biphenyls (tetrabromobisphenol-a), polychlorinated biphenyls (tetrachlorobisphenol-A), organochlorine pesticides, organophosphates, carbamates, solvents and heavy metals such as cadmium and lead, estrogenic chemicals

such as diethylstilbestrol and phytoestrogens (Biemann *et al.,* 2014; Grün & Blumberg, 2007; Janesick & Blumberg, 2011; Newbold *et al.,* 2007; Riu *et al.,* 2014; Santos *et al.,* 2012; Watt & Schlezinger, 2015), but little is known about the mechanisms originating these effects and many other potential obesogens remain to be discovered.

1.4.1 Organotins

Organotins are a class of persistent organic pollutants with potent biocidal properties that favored their use as antifouling agents in ship paints, in the production of food crops as pesticides, in industries as fungicides and as slimicides in water systems. Moreover, they were also used as wood preservatives and as heat stabilizers in the manufacture of polyvinyl chloride plastics (Grün & Blumberg, 2007; Grün & Blumberg, 2009; Lyssimachou *et al.,* 2015). Their wide range of applications, made them possible to be detected in house dust, in significant levels (Kannan *et al.,* 2010).

Obesogenic members from this class of xenobiotic chemicals include the tributyltin (TBT) and triphenyltin (TPhT) (Santos *et al.,* 2012). Within this class, the masculinizing effects of TBT became famous for causing the irreversible phenomena called “imposex”, characterized by the induction of male characteristics in female marine gastropods (Castro *et al.,* 2007). Due to its high toxicity towards many aquatic organisms and being a chemical with a tendency to be bioaccumulated, the use of TBT as an antifouling agent in paints was prohibited in 2008 (Lyssimachou *et al.,* 2015). Regardless, it is still found near ports in water samples and sediment at concentrations of 200-400 ng/L and 1-10 ng/L, respectively, as its low solubility and high octanol-water partition coefficient make this contaminant more persistent in the environment (Lyssimachou *et al.,* 2015). In 2006, Rodríguez-González and his colleagues reported levels of TBT of 1.966 µg/L in a port on the North West coast of Spain, Bay of Gijón (Rodríguez-González *et al.,* 2006), which is concerning since concentrations of TBT of 1 ng/L have been reported to be enough to cause toxicity in several marine species (Lyssimachou *et al.,* 2015; Rodríguez-González *et al.,* 2006).

The most frequent sources of organotins in general are contaminated drinking water, agricultural products and seafood (Chamorro-Garcia & Blumberg, 2014; Bašić *et al.,* 2012), resulting in measured levels of TBT, in human tissues and blood, from 0.5 nM to 27 nM, while marine mammals that bioaccumulate persistent organic pollutants reach levels of around 7 µM daily (Grün & Blumberg, 2009).

TBT was the first obesogen having his mechanism of action clarified (Janesick & Blumberg, 2011). In mammals, TBT is an RXR and PPARγ agonist and the activation of the latter is required for the obesogenic effects of this chemical (Janesick & Blumberg, 2011; Santos *et al.,* 2012). TPhT is also a high-affinity ligand of both RXRs (α, β, γ) and PPARγ,

and like TBT, it promotes the expression of adipocyte marker genes *in vitro* (Grün & Blumberg, 2009; Santos *et al.,* 2012). In fact, several *in vivo* studies report the disruption in the lipid metabolism in many tissues after exposure to these chemicals, not only in mammals but also in amphibians, teleost fish and some invertebrates (Santos *et al.,* 2012). Chamorro-Garcia and collaborators reported some transgenerational effects that *in utero* exposure to low doses of TBT has on the increase of adipose tissue in three generations of mice (Chamorro-Garcia *et al.,* 2013). TBT alters the fate of stem cells and directs them to differentiate into adipocytes (Bašić *et al.,* 2012).

Interestingly, organotins appear to have multiple modes of action. Another molecular target of organotins is the enzyme that is necessary for the conversion of androgens to estrogens, aromatase cytochrome p450, through which these chemicals inhibit the production of estradiol, thus disturbing the levels and ratios of sex steroids (Grün & Blumberg, 2007; Rodríguez-González *et al.,* 2006), which could also lead to an adipogenic effect.

Organotins were also shown to interfere with glucocorticoid homeostasis: TBT and dibutyltin cause hypercortisolism through inhibition of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) activity, the enzyme responsible for deactivating the cortisol (Grün & Blumberg, 2009; Lyssimachou *et al.,* 2015). Dibutyltin also acts as a potent antagonist of the glucocorticoid receptor (Le Maire *et al.,* 2009).

1.4.2 Thiazolidinediones

Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are pharmaceutical obesogens used to treat type 2 diabetes mellitus (Janesick & Blumberg, 2011; Santos *et al.,* 2012), by improving serum triglycerides and glycemic control (Bašić *et al.,* 2012). These synthetic drugs reduce the concentration of plasma glucose by enhancing the sensitivity to insulin in patients with low insulin responsiveness, through secretion of adiponectin (an insulin-sensitizing adipokine) and consequently increasing glucose uptake (Birsoy *et al.,* 2013; Moseti *et al.,* 2016). Since TZDs also activate the PPARγ (Moseti *et al.,* 2016; Santos *et al.,* 2012), these chemicals have the side-effects of leading to proliferation of adipocytes (increase in white adipose tissue, WAT), reduction of adipocyte hypertrophy and consequently, weight gain (Grün & Blumberg, 2009; Janesick & Blumberg, 2011; Santos *et al.,* 2012). Regardless, in this case, the diversion of calories towards adipose weight gain is considered benign, as these drugs help regularize the deleterious blood glucose levels of diabetes (Grün & Blumberg, 2009), although possible side-effects of the use for a prolonged time include increase risk of cardiovascular diseases and in the case of

rosiglitazone, increased risk of acute myocardial infarction, stroke and heart failure (Bašić *et al.,* 2012).

1.4.3 Phthalates and Perfluorooctanoic acid

Phthalates are organic additives used to increase the flexibility and durability of plastics (e.g. polyvinyl chloride) and are broadly used in food packaging, toys, paints, construction materials, electronic and medical devices and personal care products and cosmetics (Adeogun *et al.,* 2015; Janesick & Blumberg, 2011).

It seems that primary phthalates, such as (2-ethylhexyl) phthalate (DEHP), are more active on PPARα, while phthalate metabolites, such as mono[2-ethyl-hexyl]phthalate (MEHP), are selective activators of PPARγ (Grün & Blumberg, 2009). Consequently, DEHP increases fatty acid oxidation and lipid mobilization, while the presence of urinary DEHP metabolites, such as MEHP, was associated with increased waist circumference and insulin resistance in men in an epidemiological study (Grün & Blumberg, 2009; Stahlhut *et al.,* 2007).

Perfluoroalkyl compounds (PFCs) are widely used as surfactants and surface repellents in consumer products and, alongside phthalates, they are agonists of PPARs, some with a preference for PPARα, as it is the case with perfluorooctanoic acid (PFOA), thus leading to lipid mobilization, fatty acid oxidation and adipocyte atrophy (Bašić *et al.,* 2012; Grün & Blumberg, 2009). Even though on this basis it would be expected for these chemicals to reduce body weight, chronic low levels of PFOA (>5mg/kg body weight) in rodents led to increased adipose tissue mass and body weight gain after puberty (Betts, 2007).

These xenobiotic chemicals are particularly concerning due to their ubiquity in the environment (Janesick & Blumberg, 2011). PFCs leach from treated surfaces and primary phthalates from plastics, thus exposing the human being. PFC's concentration values of 0.6 ppb in drinking water, 148 ppb in surface water and a range of 4 to 133 ppb in edible fish, have been reported. Consequently, they can be detected in people serum, human breast milk and even babies' blood and the transmission through breastfeeding has been estimated to be around 200 ng/day (Betts, 2007).

In the environment, phthalates have also been detected in rainwater, surface water, treated and untreated wastewater and also sediments (Clara *et al.,* 2010), with reported values ranging from 0.2 to 50 $\mu g.L^{-1}$ in rivers (Fatoki & Vernon, 1990). The estimated average daily intake of DEHP due to transfer to food during processing is of about 160 µg per day (Tsumura *et al.,* 2003). Just like PFCs, phthalates can also be detected in human blood, urine and breast milk (Adeogun *et al.,* 2015).

1.4.4 Diethylstilbestrol

The endocrine disruptor diethylstilbestrol (DES) is a synthetic estrogen prescribed from the 40s through the 70s to pregnant women at risk of miscarriage and also to women with low levels of estrogen (Grün & Blumberg, 2007; Newbold *et al.,* 2007). It is still currently used in aquaculture to promote growth and to produce female populations of fish, contributing to its detection in surface water in a range of 1 to 20 ng/L (Lei *et al.,* 2016).

Diethylstilbestrol is a particularly interesting case of an estrogenic compound and estrogen receptor agonist that unlike other estrogens, can still have pro-adipogenic effects. Although it has been associated, in a mouse model and at concentrations of 10–100 $\mu g.kg^{-1}$ maternal body weight per day (prenatal exposure), to a decrease in offspring birth weight that was subsequently maintained, postnatal exposure to concentrations of 1 $mg.kg^{-1}.day^{-1}$, in the first 5 days corresponding to the adipocyte differentiation period, resulted in initial body weight depression, followed by a "catch-up" growth period at puberty with a subsequent increase in adult body weight, alongside a higher percentage of body fat and higher levels of serum adipokines and triglycerides (Newbold *et al.,* 2007). These results seem to show that the effects of this compound can be either pro- or anti-adipogenic, depending on the period of time in which exposure occurs (Grün & Blumberg, 2007).

1.4.5 Bisphenol A

Even though bisphenol A (BPA) is also an estrogenic compound, it is applied as a monomer in polycarbonate plastic and epoxy resins and is used widely in beverage containers, dental composites, sealants and linings of food cans, from where it can gradually leach (Bašić *et al.,* 2012; Grün & Blumberg, 2007). Hence, the human being is exposed through consumption of contaminated food, beverage and drinking water (Makinwa & Uadia, 2015), resulting in its detection in urine and other body fluids such as serum (0.2 to 4.4 ng/mL), amniotic fluid (0.2-20 ng/mL), neonatal, placenta and cord blood, and also human breast milk (Grün & Blumberg, 2009; Makinwa & Uadia, 2015).

Bisphenol A is also a widespread chemical, being found in underground water, rivers, landfill leachates, as well as air and dust. Reported environmental concentrations around the globe range from 1.9 to 3920 ng/L in river waters, 0.05 to 249 ng/L in seawaters, 0.11 to 10500 ng/g in sediments and 1 to 17400 pg/m^3 in the atmosphere (Huang *et al.,* 2012).

In the presence of insulin, BPA enhances the up-regulation of genes that promote the differentiation of 3T3-L1 preadipocytes (Grün & Blumberg, 2009). Prenatal and neonatal exposure to BPA, at concentrations similar to those detected in the human serum, led to increased body weight in rodents, along with hyperlipidemia and an apparent trend towards

increased food intake and decreased exercise (Bašić *et al.,* 2012; Grün & Blumberg, 2009), which suggests that BPA interferes with glucocorticoid metabolism, as it is able to bind to the glucocorticoid receptor (GR) (Pereira-Fernandes *et al.,* 2013).

As reviewed by Rancière et al., (2015), many studies in the epidemiological literature report a relation between BPA exposure and cardiometabolic disorders such as diabetes, hyperglycemia, cardiovascular disease, hypertension, as well as increased weight and waist circumference (abdominal obesity) in adults and children (Rancière *et al.,* 2015).

The mode of action of this chemical is not yet fully understood, as it seems possible that the activation of the nuclear estrogen receptor is not the only mechanism mediating these effects (Bašić *et al.,* 2012; Grün & Blumberg, 2009). In fact, this chemical was shown to interact with thyroid hormone receptors (Bašić *et al.,* 2012) and PPARγ (Pereira-Fernandes *et al.,* 2013).

1.5. Aim of the study

Understanding the complex network that regulates the lipid homeostasis and adipogenesis, as well as elucidating the mode of action of these obesogens and their role in the obesity epidemic, are crucial steps for the development of strategies, pharmaceuticals and therapies aiming at the alleviation of obesity.

For this reason, we have combined molecular and biochemical techniques, as well as *in vivo* assays to aid in filling some gaps found in literature.

Given the important role of nuclear receptors in obesity, we performed transactivation assays using the PPARγ, RXRα and the PPARγ:RXRα heterodimer, in order to test whether suspected obesogens were agonists of these receptors. Since *in vitro* assays and the activation of PPARγ:RXR are not enough to fully predict the obesogenic potential of a chemical, we have included an *in vivo* assay with *Danio rerio* to verify the impact of the suspected obesogen on lipid accumulation using Nile Red staining, followed by an evaluation of the expression of key lipogenic and adipogenic genes, via quantitative real-time Polymerase Chain Reaction (qPCR). These methodologies could help uncover prime targets for new drugs.

Another objective of our study is to evaluate the ability of complex environmental mixtures to deregulate metabolic pathways and induce lipid accumulation and adipogenesis. Since more than 95% of all toxicological research is focused on the effects of single chemicals (Biemann *et al.*, 2014), adding to the fact that chemical influence on obesity is an emerging topic in the scientific community, little is known about the impact of complex mixtures, and more specifically environmental samples, on obesity and related

disorders. In order to allow a more realistic assessment of this impact on animal and human health and as real-life exposure occurs more often to a mixture of chemicals with both pro- and anti-obesogenic activities, investigating the outcomes of this exposure is essential. For this purpose, we assessed the effects of wastewater treatment plants (WWTP) influents both *in vitro,* through transactivation assays and *in vivo,* using the zebrafish as a model species.

2. Methodology

2.1. *In vivo* assay

2.1.1. Species Selection: *Danio rerio*

The zebrafish (*Danio rerio*), an omnivorous freshwater fish belonging to the cyprinid family in the Actinopterygii class, is commonly used as a model vertebrate in a variety of toxicological studies (Hölttä-Vuori *et al.,* 2010). Recently, it has been proposed as an emerging model also for the study of lipid metabolism and metabolic diseases (Hölttä-Vuori *et al.,* 2010; Landgraf *et al.,* 2017). The selection of this model species is justified by a number of reasons.

Even though laboratory rodent studies are significant for the extrapolation of data to humans, its restriction by law, higher price and time-consumption (Hill *et al.,* 2005), make this model less available. Moreover, the conservation of genes, receptors and molecular processes across animal phyla (Hill *et al.,* 2005), as well as the conservation of the regulation of energy homeostasis at both the neural and endocrine levels, when compared to the human being and the common pathophysiological pathways of diet-induced obesity shared with mammals (Birsoy *et al.,* 2013; Hölttä-Vuori *et al.,* 2010; Landgraf *et al.,* 2017), are additional justifications to show that the zebrafish model is suitable to represent more complex animals. Hence, it is not surprising that this species' organs and tissues have similar structures and functions to those of humans (Landgraf *et al.,* 2017) and that fish are able to develop metabolic diseases also reported in humans (e.g.: non-alcoholic fatty liver disease and hepatic steatosis) (Lyssimachou *et al.,* 2015).

Bioassays using zebrafish are simplified due to this species short-life cycle and also due to the fact that its ideal breeding and maintenance conditions are known and thus, can be induced to breed all year under controlled laboratory conditions (Hill *et al.,* 2005; Soares *et al.,* 2009). The zebrafish attains sexual maturation fast (around 100 days) and each pair is able to lay 200 to 300 eggs in a single morning, every 5 to 7 days (Hill *et al.,* 2005).

Moreover, being a species of small size (26 to 38 mm as an adult), it allows an increase in sample size at the same time that the housing space is reduced, minimizing the costs of

the dosing solutions and experimental chemicals, as well as the disposal of waste (Hill *et al.,* 2005).

Particularly, for our study, the optical clarity of this species is helpful for the screening of lipid accumulations stained with a fluorescent dye in very small larvae, without having to dissect the animal. Also, given the fact that the zebrafish genome is completely sequenced, it is useful for the study of altered gene expression in response to toxic insult, through qPCR and genomic screens (e.g. microarrays) (Hill *et al.,* 2005; Veldman & Lin, 2008).

2.1.2. Animal breeding

All the steps described in this section were conducted in the facilities of BOGA (Biotério dos Organismos Aquáticos – CIIMAR).

A breeding stock of adult wild-type zebrafish (*Danio rerio*; Singapore) was kept in an aquarium with 250L of capacity filled with dechlorinated aerated water, at a temperature of 28 ± 1°C, under a photoperiod of 14:10h (light:dark). The fish were fed with commercial diet Tetramin (Tetra, Melle, Germany) twice a day.

In the afternoon before zebrafish breeding, adult wild-type zebrafish (9 females: 9 males) were placed in cages within a 30L aquarium, under the same water and photoperiod conditions as the stock from which the breeders were selected, and were fed once with commercial fish diet Tetramin. The cages possess a net bottom cover, covered with glass marbles to prevent eggs from being consumed by adults.

In the subsequent morning, following the collection and cleaning of the eggs, the transparent ones were selected and randomly allocated to different experimental aquaria, assuming that the transparency was indicative of the viability and fertilization of the eggs.

2.1.3. Test conditions and water parameters

The embryos obtained by natural mating were raised in 3L aquaria filled with dechlorinated water, at 28 ± 1 °C with a 14L:10D photoperiod. Each aquarium contained a total of 80 eggs.

From hatching (4th day post-fertilization) until the end of the experiment (18th day post-fertilization), the larvae were exposed to six different treatments under semi-static conditions, each set in duplicates: experimental control, solvent control (0.0002% dimethylsulfoxide, DMSO), TBT at 100 ng/L Sn and 200 ng/L Sn, and WWTP influent at 1.25% and 2.5%.

The TBT test solutions were obtained by successive dilutions of a stock solution containing TBT chloride (96%, Sigma-Aldrich) in DMSO (99.9%, Sigma-Aldrich), and were

all kept at -20°C until use. Calculations were made in order to ensure that each aquarium, with the exception of those from experimental control, had the same DMSO concentration as the aquaria from solvent control.

In order to determine the actual concentrations in the aquaria and the stability of the compounds, water samples were collected once during the assay, at the moment right before water renewal and about 20 minutes after the contamination of the new water.

The medium was renewed daily by changing at least two thirds of its volume, while retaining the larvae in the aquaria. The cleaning procedures consisted of rubbing the walls and syphoning the bottom of the aquaria.

The ammonia levels were checked weekly at 640 nm (0.3625 ± 0.033 mg/L N and 0.4375 ± 0.0369 mg/L N for the 1.25% and 2.5% WWTP influent, respectively, and 0.1425 ± 0.0347 mg/L N for all remaining treatments), using Palintest tablets and Palintest Photometer 7000se, while the temperature was monitored daily, with individual thermometers in each aquarium. Values of mortality were registered daily (total mortality below 20% in all aquaria) and the dead larvae/eggs were removed.

A light aeration was introduced to the aquaria on the 11th day post-fertilization of larvae (Figure 2).

Throughout the whole experiment, care was taken in order to avoid the suffering and distress of the animals.

Figure 2. *In vivo* assay with zebrafish. The aquaria from each treatment were randomly allocated to the water baths. All three water baths contained 2 electric resistances (image on the right). Each aquarium contained its own aeration system.

2.1.4. Feeding protocol

From 5 days post-fertilization (dpf) to 14 pdf, larvae were fed with a standard diet, Gemma Micro (Skretting; 59% protein, 14% oil), supplemented with 25% of additional lipids.

For the diet preparation, the standard diet was ground to powder and mixed with an emulsion of cod liver oil (Fagron) in a mixer to form a homogeneous moist blend. The volume of cod liver oil added to the original diet was calculated in order to obtain a dietary lipid content of 25% dry matter in the final diet. The blend was pelleted and pellets were dried in an oven at 40ºC for 24 h. Dried pellets were crushed and sieved through a battery of sieves to obtain food particles with a diameters of 100-200 and 200-400 µm.

Excluding the period between the 15th and the end of the assay, the zebrafish larvae were fed *Artemia spp.* nauplii (Brine Shrimp Eggs; Ocean Nutrition) every other day (put to hatch for 16 hours from the 7th to the 11th day postfertilization and 24 hours on the 13th day). For this end, 0.2 grams of *Artemia spp.* cysts were incubated in a reactor with 1L of warm water (1 part of seawater for 4 parts of freshwater at 28 °C) and this quantity was used to feed 3 aquaria with 80 larvae each. Aeration was provided to the reactor in order to maintain the oxygen levels and to keep cysts suspended in the water column. A bright light was also delivered to maximize hatch rates.

On the 15th and 16th day postfertilization, the larvae were fed lyophilized chicken egg yolk (produced at the laboratory in CIIMAR) as a high fat diet, and were finally followed by one day of starvation prior to analysis on the 18th day postfertilization.

The specifications of the feeding regime are summarized in table 1.

Table 1. Feeding regime throughout the assay.

			Period	(dpf)	
	0 to 4	5 to 11	12 to 14	15 to 16	17 to 18
Feeding type	not fed	Gemma Micro 100-200 µm*	Mixture Gemma 100-200 µm and 200-400 µm*	lyophilized egg yolk and Gemma 200-400 µm	fasting state
Quantities	None	12 mg (3 times a day)	24 mg (100-200 µm twice a day and 200-400 µm once)	egg yolk 40 mg (once) and Gemma 24 mg (twice)	None

* From the 7th day postfertilization, the larvae were also fed every other day with *Artemia* spp. with 16h since eclosion. On the 13th day, the larvae were fed with *Artemia* spp. with 24h.

2.1.5. Nile Red staining

On the day of the analysis (18th day postfertilization), 10 larvae of similar size (6.55 ± 0.56 mm) were selected per aquarium and individually incubated with Nile Red (Sigma-Aldrich) in 3mL-wells plates, for 60 minutes, at 28 °C and in the dark, in order to avoid photobleaching. For this, a Nile Red stock solution of 5 mg per mL of acetone (Biochem, Chemopharma) was prepared and diluted 1:1000 in dechlorinated water.

After the incubation, the wells were rinsed twice with dechlorinated water and anaesthetized during 30 seconds with Tricaine 1000mg/g (Pharmaq), stock of 168 mg/mL diluted 1:1000 in dechlorinated water.

The larvae were analyzed in water within approximately 2 minutes, under the microscope Nikon Eclipse TS100 with a Nikon Intensilight C-HGFI unit, while using a fluorescence filter (B-2A: EX: 450-490nm, BA: 520nm).

All images were captured under the same settings with imaging software NIS-Elements D (version 4.13) and saved in high-resolution (1280x960) Joint Photographic Experts Group (JPEG) format. The total length, which is the distance from the rostral tip to the tip of the longer lobe of the caudal fin, was registered in the examined larvae.

The fluorescence signals were subsequently determined using ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij/). The procedure for these adiposity recordings begin with the conversion of the images into 8-bit grayscales, followed by the measurement of background fluorescence (calculated individually in each image), in areas that did not contain any adipocytes. The value of larvae fluorescence was obtained by selecting the white adipose tissue and individual adipocytes in the perivisceral area (Figure 3) with the “polygon selections” tool of the computer program, and subtracting the background fluorescence. The values shown in the graph of results, expressed as the average fold changes ± standard error of the mean (SEM) of the solvent control group, match the values of the Integrated Density (the product of area and mean gray value), obtained in the software for each larvae.

At the end of the bioassay, the remaining zebrafish were euthanized in MS-222 (Ethyl 3-aminobenzoate methanesulfonate 98%; Sigma-Aldrich) at a concentration of 200 mg/L in order to guarantee minimum pain. 20 of these larvae (per aquarium) were then stored in

RNAlater (Sigma) for 24h at 4°C and then moved to -80°C, for posterior molecular biology determinations.

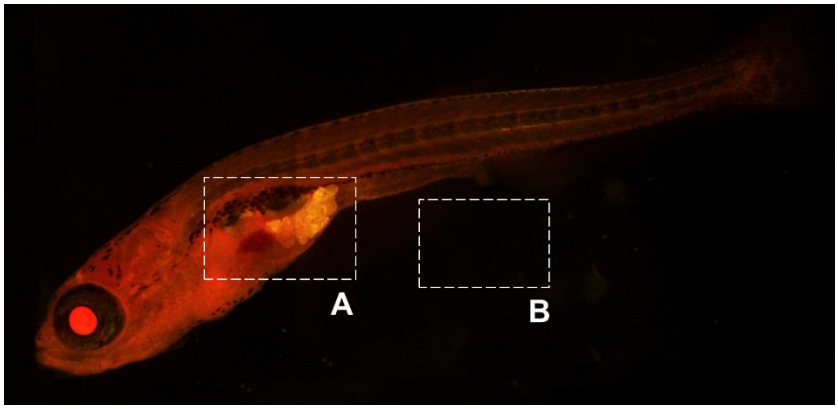

Figure 3. Zebrafish at the 18th day postfertilization colored with Nile Red dye; A: Perivisceral area from which the measurements of WAT and individual adipocytes were taken; B: Example of an area for background fluorescence (note: image not converted to 8-bit grayscales).

2.2. Evaluation of gene expression

2.2.1. RNA extraction and cDNA synthesis

The total RNA was extracted from each body of zebrafish larvae, separately, while the heads were kept in RNAlater at -80°C for future research.

The RNA extraction was performed using illustra™ RNA spin Mini RNA Isolation kit (GE Healthcare), according to the kit's instructions. For this purpose, samples were firstly homogenized and lysed in tubes containing 350 µL of RA1 (lysis buffer) from the kit, 3.5 µL of β-Mercaptoethanol (Merck) and 2 beads. The tubes were then homogenized using the Precellys®24 lysis & homogenization equipment (Bertin Technologies), for 2 x 10 seconds at 6500 rpm.

At the end of each extraction, the RNA quality was confirmed in a 1% agarose (NZYtech) gel, run in 1x of Tris-Acetate-EDTA buffer (TAE 50x; Bio-Rad) for 25 minutes at 100V and containing 1µL GelRed™ (Biotium) for visualization of bands under UV light. The concentration of this nucleic acid was determined using Gen5™ software and equipment Synergy HT Multi-Mode Plate Reader (BioTek), where each sample was read in 2 replicates. RNA samples were then kept at -80 °C until use.

For the cDNA synthesis, the iScript™ cDNA Synthesis (Bio-Rad) kit was used, following the kit instructions and 200 ng of RNA was loaded in each reaction tube. The synthesis was performed using the TGradient Thermocycler (Biometra), then the cDNA samples were kept at -20°C until their use in Real-time PCR.

2.2.2. Real-time PCR

In order to evaluate the expression of target genes involved in lipid homeostasis and adipogenesis, fluorescence-base quantitative (real-time) PCR was performed.

Gene sequences were retrieved from the National Center for Biotechnology Information (NCBI) and primers were designed using the Primer-Blast-NCBI tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The selected pair of primers was then synthesized by STABVIDA (Portugal). The primers and respective conditions are listed in table 2.

Table 2. Primer pair sequences and respective $T_{annealing}$ used in real-time qPCR analysis.

Gene	Genbank access number	Forward primer (5´- 3´)	Reverse primer (5´- 3´)	$T_{annealing}$ (°C)
RXRα	NM_001161551	ATTCAATGGC´ATCTCC TG	GCGGCTTAATATCC TCTG	60
PPARγ	NM_131467	GGTTTCATTACGGCGT TCAC	TGGTTCACGTCACT GGAGAA	60
SREBP1	NM_001105129.1	CAGAGGGTGGGCATG CTGGC	ATGTGACGGTGGT GCCGCTG	60
FASn	XM_682295	ATCTGTTCCTGTTCGA TGGC	AGCATATCTCGGCT GACGTT	62
ACOX1	BC097101.1	GCACGGATGTGTGTAC CGTGC	GCGTCCAGAGCCC CTTGACCT	62
β-actin	NM_181601.3	ACTGTATTGTCTGGTG GTAC	TACTCCTGCTTGCT AATCC	60

The expression of reference gene β-actin and target genes PPARγ, RXRα, FASn, ACOX1 and SREBP1, individually, was assessed for each body of larvae separately, using the Mastercycler ep realplex system (Eppendorf). Each sample was amplified in duplicate and loaded into 96-well white Multiplate™ PCR plates (Bio-Rad). Each well contained 10 µL of reaction volume, corresponding to 1 µL of cDNA (equals 10 ng), 5 µL of IQ ™ SYBR® Green Supermix (Bio-Rad), 200 nM of each primer and ultrapure water. The reaction was performed according to the Taq polymerase's protocol, which consisted in a two-step real-time PCR program with an enzyme activation step at 95°C (3 min), followed by a 40-cycles amplification starting with a denaturation step at 95°C for 10 seconds, then annealing/extension around 60°C (1 min). A melting curve (55-95°C with 0.5°C increment) was also generated at the end in order to confirm the specificity of each amplification. The products from the PCR reactions were then visualized in a 1% agarose gel, to further check whether or not non-specific products were amplified, by the presence of single bands.

A standard curve using successive dilutions of cDNA pools from each sample (6 dilution step at a dilution factor of 1:5) was created to determine the efficiency of the reactions for each gene. Efficiencies between 88-109% were considered acceptable.

The β-actin reference gene was validated by its stability among the exposure groups and controls, verified through one-way ANOVA.

Since the expression of all genes was evaluated for each fish individually, comparisons among genes in the same individual could be achieved.

The relative quantification of target genes was performed according to the Livak method (Livak & Schmittgen, 2001). The relative changes in gene expression of target genes were calculated based on the amount of target normalized to β-actin and relative to an internal calibrator:

$$\text{Amount of target} = 2^{-\Delta\Delta Ct} = (2^{-(Ctsample-Ctcalibrator)_{target}}) / (2^{-(Ctsample-Ctcalibrator)_{reference}})$$

The final data were expressed as fold changes of the solvent control group: mean values of treatment dividing by the mean value of solvent control group.

2.3. Transactivation assays

2.3.1. Preparation of RXRα and PPARγ constructs

Danio rerio RXRα and PPARγ sequences were collected from the GenBank database using accession codes XM_005161184.2 and NM_131467.1, respectively, and primers were designed to amplify the portion of the gene correspondent to the Hinge Region (HR) plus Ligand Binding Domain (LBD) of each receptor (Table 3), using the Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/). Additional oligonucleotides of the recognition sequence for the restriction enzymes XbaI and KpnI were also added to the primers.

Table 3. Primers designed to amplify the HR+LBD of the NRs. The forward primer contains the recognition sequence of XbaI, while the reverse primer contains the sequence for KpnI.

NR	Forward Primer (5´- 3´)	Reverse Primer (5´- 3´)
RXRα	ATCGTCTAGACAGCGAGCCAAGGAACGC	AATTGGTACCTTATGTCATTTGGTGTGGAGCTT
PPARγ	ATCGTCTAGACTGGCCGAGTTCTCCAGT	AATTGGTACCTTAGTACAGGTCCCGCATGA

The amplification was performed through Rapid Amplification of cDNA Ends (RACE) PCR, using *Danio rerio* cDNA pool as a template for RXRα and *Danio rerio* liver cDNA for

PPARγ. The resulting products were run on a 1% agarose gel and purified using NZYGelpure (NZYTech) kit. Each sequence was then digested with the restriction enzymes XbaI and KpnI (Promega), for insertion of RXRα and PPARγ in a pBIND vector and for insertion of RXRα in a pACT vector, followed by a new purification using the kits aforementioned. The T4 DNA Ligase (Promega) was used to insert the DNA into the pBIND or pACT vectors, previously digested with the same restriction enzymes and dephosphorylated using an alkaline phosphatase (NZYtech) in accordance to the product brochure. The constructs were sent to sequence in order to ensure the integrity and orientation of each sequence in the expression vector.

Escherichia coli competent cells (NZY5α, NZYTech) were transformed with the ligation product of each reaction. The cells were plated on agar covered with ampicillin (20 µL per petri disk), where they were grown in an inverted position for 16h at 37°C, and a colony screening, using pBIND primers or pACT primers and NZYTaq 2× Green Master Mix (NZYTech) on the TGradient Thermocycler (Biometra), was performed to assess which colonies expressed the vector with the insert (positive colonies), by running the resulting products in another electrophorese gel. Subsequently, these colonies were selected for the growth of minicultures in LB medium (LB Broth powder, NZYTech) supplemented with 0.1% ampicillin (NZYTech), and minipreps were prepared using the NZYMiniprep kit (NZYTech). Each miniprep was sequenced to check if any mutations occurred during the procedure. From the miniculture, 400 µL were collected to a 2 mL tube and 100 µL of glycerol (≥99%, Sigma-Aldrich) were added to create a glycerol stock.

Afterwards, midicultures were grown and midipreps were prepared using the NZYMidiprep kit (NZYTech). The DNA was quantified using Gen5™ software and equipment Synergy HT Multi-Mode Plate Reader (BioTek), and the samples were stored at -20 °C for posterior use in the transactivation assays.

The *Homo sapiens* RXRα and PPARγ were kindly provided by laboratory colleagues (groups EDEC and AGE) who already used them in their experiments. The procedures used in the acquisition of these genes were analogous to the above mentioned.

2.3.2. Fractionation of WWTP samples

Water samples from the influent and post-UV treatment were collected from Sobreiras WWTP located in a residential area near the Douro's river estuary in Porto, Portugal, on the 26th of April 2016. 500mL samples, each with 5 replicates, were collected during a week and stored at -20 °C until use. Three of these replicates where sent for chemical analysis at the University of Santiago de Compostela. The remaining two were fractionated for use in transactivation assays.

For the fractionation, the collected samples were previously filtered and the pH values were measured. Oasis WCX and WAX (Waters) solid-phase extraction cartridges were conditioned with 5mL of methanol/formic acid (98:2) and ammonia solution 7N in methanol, respectively, followed by 5mL of methanol and 5mL of MiliQ water. The cartridges were then connected in series, where WAX was put on top. 100mL of each sample were percolated through the connected cartridges, followed by a washing step with 10 mL of MiliQ water.

After a 30 min drying under vacuum, four fractions were collected with the following elution conditions designed to guarantee the maximum yield possible of putative nuclear receptor ligands: fraction A – 10mL of methanol (100%, VWR International); fraction B – 10mL of ammonia solution 7N in methanol (Sigma-Aldrich); fraction C – 10mL of methanol/formic acid (98%, Merck) 98:2; fraction D – 10mL of ethyl acetate (99.7%, Sigma-Aldrich). The obtained fractions were then blown to dryness under a nitrogen steam and re-dissolved in DMSO, prior to use in transactivation assays, reaching a concentration of 200x the pure WWTP sample.

2.3.3. Transactivation assay

To characterize the RXRα, PPARγ and the heterodimer RXRα:PPARγ response to different compounds, the plasmids expressing the NR LBD fused with the yeast GAL4 DNA Binding Domain (DBD) were transfected into COS-1 cells (kidney, *Chlorocebus sabaeus*). This procedure consists on the binding of the GAL4 DBD to the promoter of the luciferase gene and the determination of the quantity of the enzyme produced. If the LBD of the NR under test is transactivated, the transcription of luciferase is increased and its concentration can be determined from its luminescence when a substrate of the enzyme is added.

On the first day, viable COS-1 cells were counted in the Countess™ Automated Cell Counter (Invitrogen) using Trypan Blue Stain (0.4 %; Invitrogen), and distributed in a 24 wells-plate, at a density of approximately 2×10^5 cells.mL^{-1}, where they were grown in DMEM with phenol red (PANBiotech) supplemented with 1% Penicillin/Streptomycin (PANBiotech) and 10% fetal bovine serum (PANBiotech), for 24h at 37 °C and 5% CO2.

The confluence of the cells (considered optimal around 80%) was checked on the following day under a microscope and the transfection was performed. For this procedure, a mix with Lipofectamine (Invitrogen) and Opti-MEM 1x (Gibco) was prepared (2µL lipofectamine + 23µL of Opti-MEM per well), as well as a mix with the testing plasmids, ensuring that each well has 500 ng of pBIND, 500 ng of pGAL4 and 750 ng of pcDNA3 (or pACT on the heterodimer assays). The two solutions were incubated separately for 5 min and then mixed and incubated at room temperature for another 20 min. The following step

consisted in preparing the transfection solutions by diluting each solution mix in Opti-MEM to perform a total volume of 350 µL/well. For this purpose, the DMEM was firstly removed from the plate, then the cells were washed with a DPBS solution (PANBiotech) and finally, the 350 µL of the transfection solution were added to each well and the cells were incubated for 5h at 37 °C and 5% CO2.

Following the 5h of incubation, *Danio rerio* and *Homo sapiens* RXRα, PPARγ and the heterodimer RXRα/PPARγ were exposed to different treatments: DMEM without phenol-red supplemented with 1% Streptomycin/Penicillin and 10% charcoal-treated Fetal Bovine Serum (PANBiotech) was contaminated with DMSO 0.1% (solvent control; 99.9%, Sigma-Aldrich), TBT 250 nM (positive control; TBT chloride 96%, Sigma-Aldrich) and several WWTP samples (3 dilutions of pure WWTP sample, 1.25%, 2.5% and 10%, and the 4 fractions of influent WWTP sample, fractions A, B, C and D, at a concentration of 1 µL of concentrated solution to 999 µL of DMEM without phenol-red). The transfection medium was removed from each well, the cells were washed with DPBS solution and were exposed to the different concentrations, followed by a new incubation for 24h at 37°C and 5% CO2.

On the following day, cells were lysated with the Passive Lysis Buffer 5× (Promega) diluted in distillated water to a concentration of 1x and incubated at 37°C for 15 min at 90 rpm. For the luciferase activity detection, the Dual-Luciferase® Reporter Assay System (Promega) was used. The luminescence was measured with the equipment Synergy HT Multi-Mode Plate Reader (BioTek), using the Gen5™ software.

At least three replicates were performed for each transactivation assay in order to guarantee a correct statistical treatment of the data.

2.4. Statistical analysis of data

The statistical analysis was performed on IBM SPSS Statistics 24 software, where the normality and homogeneity of variance of all data was confirmed using Shapiro-Wilk and Levene's tests, respectively, prior to analysis through one-way ANOVA.

Subsequently, the Nile-Red fluorescence data and real-time PCR results were further analyzed through Fisher's Least Significant Difference (LSD) post hoc test, while transactivation data were examined by Bonferroni post hoc test. *p*-values lower or equal to 0.05 were considered statistically significant.

When parametric assumptions were not attained, the PCR and transactivation data were log-transformed and Nile-Red data was square-rooted.

All values were expressed as the average fold changes ± standard error of the mean (SEM) of the solvent control group.

3. Results

3.1. *In vivo* assay

The results from the *in vivo* assay displaying lipid accumulation, analyzed through the fluorescent dye Nile-red, are presented in Figure 4.

The values obtained from the Image J software correspond to the Integrated Density (the product of area and mean gray value) which represents the fluorescence captured for each larvae. Hence, the higher the values of Integrated Density, the higher the accumulation of lipids in larva.

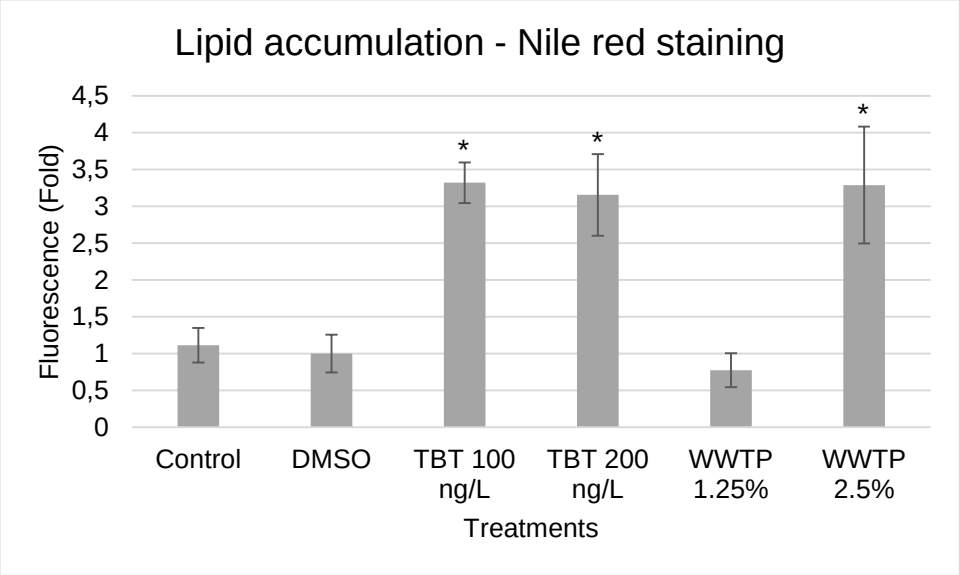

Figure 4. *In vivo* induction of lipid accumulation after exposure to TBT and WWTP influents. Values were expressed as the average fold changes ± SEM of the solvent control group. *$p < 0.05$ compared to solvent control (one-way ANOVA, followed by Fisher LSD's test).

The graph shows a clear increase in lipid accumulation of larva exposed to 100 ng/L Sn TBT, 200 ng/L Sn TBT and WWTP influent 2.5%.

Interestingly, the lowest concentration of WWTP influent tested, 1.25%, did not show any augmentation of lipid accumulation, in opposition to the observed results at the highest concentration.

3.2. Real-time qPCR

Figure 5 displays the effects of TBT and WWTP influents on the expression of key transcription factors and metabolizing enzymes, involved in adipogenesis and lipogenesis, in the bodies of zebrafish larvae: RXRα, PPARγ, SREBP1, ACOX1 and FASn.

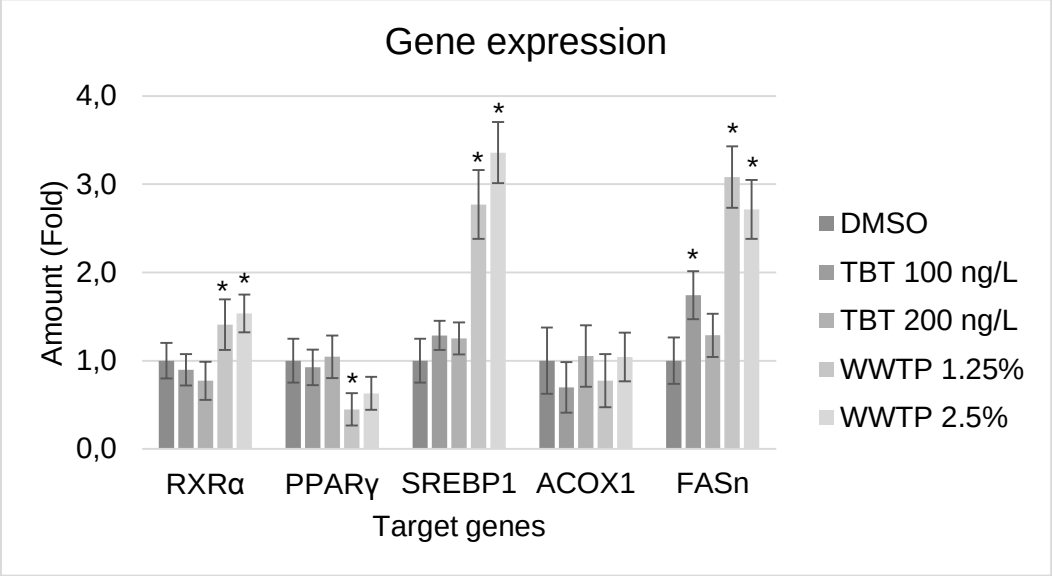

Figure 5. *In vivo* induction of lipogenic and adipogenic genes in zebrafish, following exposure to TBT and WWTP influents. Values initially normalized to β-actin and relative to an internal calibrator are expressed in the graph as the average fold changes ± SEM of the solvent control group. *$p < 0.05$ compared to solvent control (one-way ANOVA, followed by Fisher LSD's test).

Chronic exposure of zebrafish to the environmentally relevant concentrations of TBT (100 ng/L Sn and 200 ng/L Sn) did not affect the expression of transcription factors RXRα, PPARγ and SREBP1.

In contrast, the expression of key lipogenic gene fatty acid synthase (FAS) was significantly up-regulated at the lowest TBT concentration, of 100 ng/L Sn.

In regard to the WWTP influent treatments, all adipogenic and lipogenic genes were significantly changed after exposure, with up-regulations detected for RXRα, SREBP1 and FASn at all concentrations, and a statistically significant down-regulation of PPARγ at the lowest concentration tested (influent at 1.25%).

The expression of acyl-CoA oxidase (ACOX), the enzyme involved in peroxisomal β-oxidation, was not affected by any of the treatments.

3.3. Transactivation assays

For the evaluation of a ligand/mixture's ability to transactivate the nuclear receptors *in vitro*, the Dual-Luciferase® Reporter Assay System (Promega) was used. This method allows the measurement of the activity of 2 different luciferases, firefly luciferase (*Photinus pyralis*) and *Renilla* luciferase (*Renilla reniformis*), the latter being required as an internal control for the normalization of the data, since differences in amounts of cells between wells would lead to different levels of luminescence. Finally, the results were expressed as average fold changes of the solvent control.

Graphical representations of the results of the transactivation of RXRα, PPARγ and the heterodimer are presented in Figure 6, Figure 7 and Figure 8, respectively.

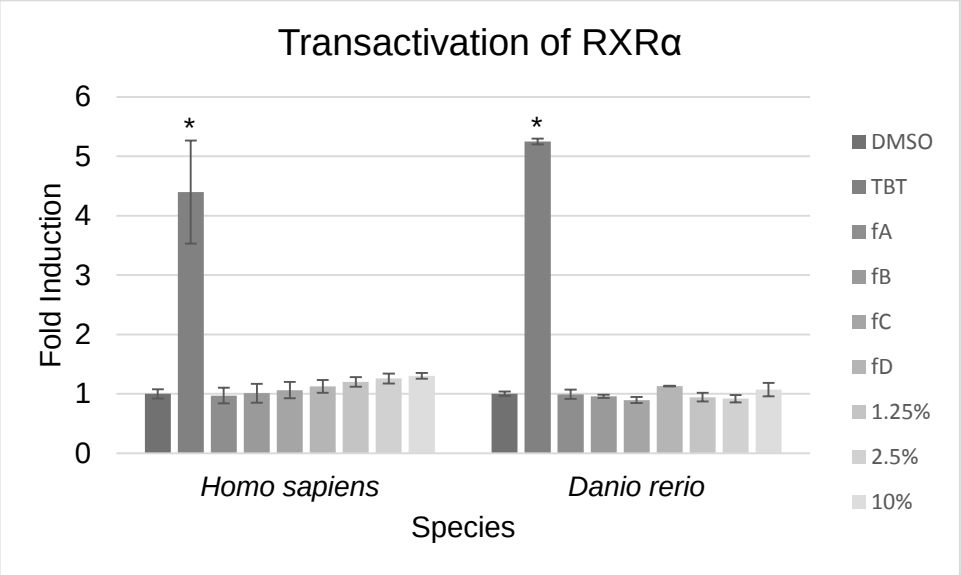

Figure 6. *In vitro* transactivation of *Homo sapiens* and *Danio rerio* RXRα by TBT and WWTP influents. The activity of firefly luciferase (*Photinus pyralis*) was normalized to the activity of *Renilla* luciferase (*Renilla reniformis*) and then expressed as average fold changes ± SEM of the solvent control group. *p < 0.05 compared to solvent control (one-way ANOVA, followed by Bonferroni test).

The results from the assays with RXRα indicate that only our positive control (TBT at a concentration of 250 nM) was able to transactivate this receptor in both the *Homo sapiens* and the zebrafish.

In regard to the WWTP influents, neither the fractions nor the pure influent caused an effect on the nuclear receptor. Although for the human RXRα, a slight dose-dependent increase seems to exist, these results were not considered statistically significant through one-way ANOVA.

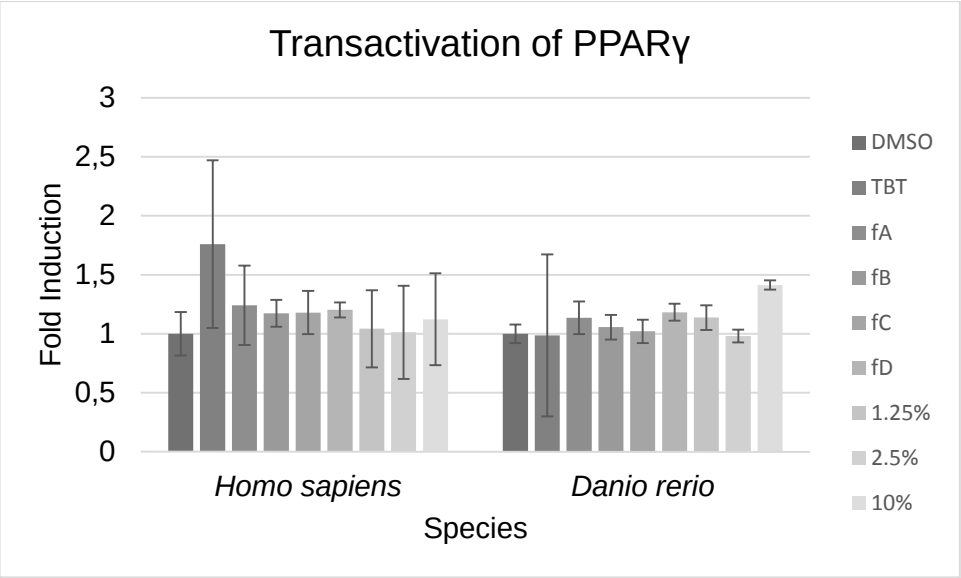

Figure 7. *In vitro* transactivation of *Homo sapiens* and *Danio rerio* PPARγ by TBT and WWTP influents. The activity of firefly luciferase (*Photinus pyralis*) was normalized to the activity of *Renilla* luciferase (*Renilla reniformis*) and then expressed as average fold changes ± SEM of the solvent control group. *p < 0.05 compared to solvent control (one-way ANOVA, followed by Bonferroni test).

Surprisingly, TBT at 250 nM did not significantly enhance the transcription of the firefly luciferase gene by the PPARγ, even though there appears to be a slight induction of the *Homo sapiens* receptor.

Likewise, both the fractions and the pure WWTP influent did not transactivate this receptor in either species. The concentration of 10% of pure influent appears to lead to an induction of the *Danio rerio* PPARγ activity, yet not considered statistically significant.

Taking the results from both transactivation of RXRα and PPARγ together, it is possible to conclude that TBT is a far more powerful agonist of the RXRα in both species, in comparison to PPARγ.

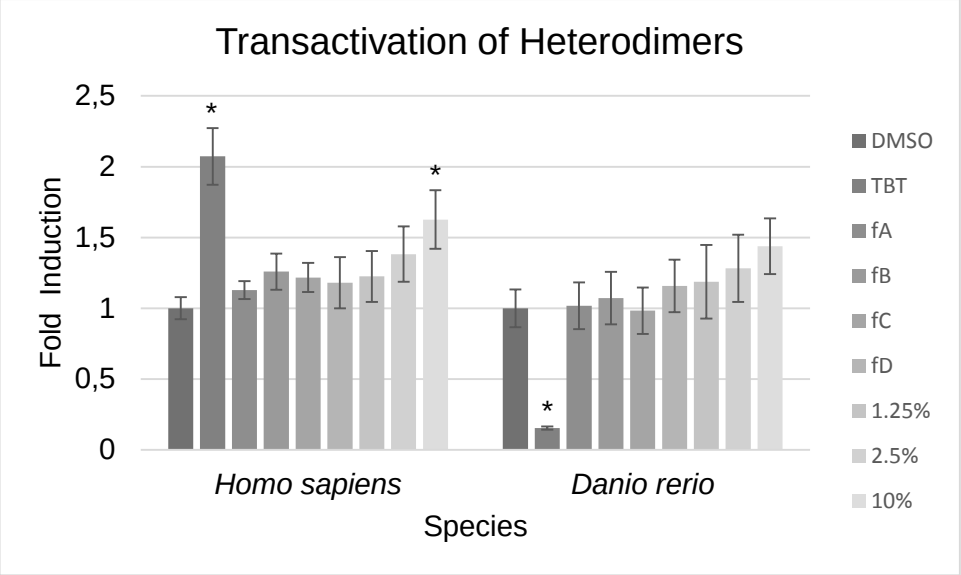

Figure 8. *In vitro* transactivation of *Homo sapiens* and *Danio rerio* PPARγ:RXRα heterodimer by TBT and WWTP influents. The activity of firefly luciferase (*Photinus pyralis*) was normalized to the activity of *Renilla* luciferase (*Renilla reniformis*) and then expressed as average fold changes ± SEM of the solvent control group. *p < 0.05 compared to solvent control (one-way ANOVA, followed by Bonferroni test).

The heterodimer transactivation results show that TBT does influence the activity of both *Homo sapiens* and *Danio rerio* PPARγ:RXRα heterodimers. However, the zebrafish heterodimer follows an inverse tendency when compared to the human heterodimer, as there is a massive repression at the tested concentration. Moreover, the response of this heterodimer contrasts with the response of the individual nuclear receptors, since a significant transactivation was detected for the RXRα exposed to TBT.

The WWTP influent fractions did not transactivate the heterodimers, yet the pure influent appears to lead to a dose-dependent increase in transactivation, yet only statistically significant at a concentration of 10% for the *Homo sapiens* heterodimer.

4. Discussion

It has become clear that obesity is not simply the result of overeating and sedentary lifestyles, but instead, the product of a variety of internal and external factors, from which chemical exposure might play an important role.

The study of lipid accumulation and adipogenesis from lower organisms to mammals has helped increase our knowledge of the molecular mechanisms behind lipid homeostasis and adipocyte formation. Regardless, many questions remain to be answered.

Even though many important features in the regulation of lipid homeostasis are conserved throughout metazoans, the large majority of studies have focused on the effects of obesogens in mammalian models (Capitão *et al.,* 2017). Thus, for a better understanding of the effects and possible mechanisms of action of these obesogens across vertebrates as well, we have combined molecular and biochemical techniques, alongside with an *in vivo* assay with the vertebrate model species *Danio rerio*. Particularly, we addressed two key questions: What is the molecular mode of action of suspected obesogens? Are complex environmental samples able to induce an obesogenic response in the zebrafish?

Our study demonstrates that exposure to environmental mixtures and a model obesogen, TBT, at environmentally relevant concentrations, induces mechanisms that favor adipogenesis and lipogenesis both *in vivo* and *in vitro*.

Exposure of *Danio rerio* from hatching to the 18th day postfertilization to TBT at 100 ng/L Sn and 200 ng/L and to WWTP influent at a concentration of 2.5%, resulted in a significant accumulation of lipids, analyzed through Nile red staining. The Nile red is a fluorescent dye that acts as a hydrophobic probe and makes lipids fluoresce in tones of yellow to red depending on the degree of their hydrophobicity (Fowler & Greenspan, 1985), allowing the visualization and quantification of the fluorescence that is indicative of the lipid accumulation in the different treatments.

Lipid staining techniques have been used several times to determine changes in lipid content following exposure to obesogens. Tingaud-Sequeira *et al.,* (2011) presented the Zebrafish Obesogenic Test (ZOT) as an intermediate step in obesity research between *in vitro* and rodent assays. Their short-term assay method consisted in a 3-day treatment protocol with adiposity recordings prior and posterior to chemical exposure. The zebrafish larvae were reared on a standard diet until the first day of the protocol where two separate groups were fed for a day with different types of diet (standard diet or hard-boiled chicken egg yolk as a high-fat diet), followed by two days of starvation. After the first day of starvation, the initial adiposity was recorded and larvae were posteriorly exposed for 24 h to 50 nM TBT and 1 nM rosiglitazone, followed by a final adiposity recording. Similarly to our study, lipid droplets were analyzed through Nile red staining and a quantitative analysis the fluorescence was performed. The results indicated that the use of high-fat diets leads to a more dramatic increase in lipid adiposity when the fishes were exposed, as noted in the treatment with rosiglitazone which demonstrated the additive effect of the diet and chemical exposure. Additionally, TBT exposure led to adipocyte hypertrophy irrespective of the type of background diet while the larvae were in a fasting state (negative energy

balance), confirming that it has a strong obesogenic effect, even at environmentally-relevant concentrations.

Zebrafish larvae exposed daily, from 3 dpf to 11 dpf, to tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) both at 10 and 100 nM, TBBPA-sulfate (TBBPA-S) at 125 and 250 nM or TBT at 0.1 and 1 nM, and fed with an egg yolk diet from the 6th to 10th dpf, exhibited a significant increase in lipid accumulation assessed by Oil Red O staining. The authors also pointed out that when the larvae were fed with a regular diet, instead of one with high-fat content, none of the treatments resulted in the staining of larvae, thus highlighting the impact of the synergy of both high-caloric diets and chemical exposure in obesity. Moreover, larvae exposed to relatively low concentrations of 100 nM TBBPA, 100 nM TCBPA or 1 nM TBT from 3 to 11 dpf, fed with egg yolk diet, and then put in a chemical-free housing system and fed with regular diet until 30 dpf, had a significantly higher body mass index, which demonstrates that exposure to halogenated BPAs during early development can lead to late-onset weight gain (Riu *et al.,* 2014).

3T3L1 preadipocytes treated with a mix of insulin with obesogens rosiglitazone (100 nM to 1 µM) or MEHP (monoethyl-hexyl-phthalate; from 10 µM to 100µM) for 10 days exhibited, in a dose-dependent manner, an induced adipogenic differentiation and increased triglyceride accumulation, analyzed through Oil Red O staining, whereas the parent compound DEHP (diethyl-hexyl-phthalate) did not influence adipogenesis (Feige *et al.,* 2007).

Watt and Schlezinger (2015) treated bone marrow mesenchymal stem cells of 9-week-old male C57BL/6J mice with rosiglitazone (100 nM), TBT (100 nM), triphenyltin (TPhT; 10, 50 and 80 nm), MEHP, mono-(2-ethylhexyl) tetrabromophthalate (METBP), or TBBPA (10 and 20 nM) and reported increases in lipid accumulation for all the toxicants tested, assessed through Nile Red staining (Watt & Schlezinger, 2015).

In the zebrafish, lipids are mostly stored in the form of triacylglycerols in visceral, intramuscular and subcutaneous white adipose tissue (WAT) depots, with the first appearance being associated with the pancreas following exogenous nutrition from 8 to 12 dpf or at a minimal size of around 5 mm (Hölttä-Vuori *et al.,* 2010; Riu *et al.,* 2014).

The increase in white adipose tissue around visceral organs, as we found in our assay, is precisely the one related to obesity (Chamorro-Garcia & Blumberg, 2014; Moseti *et al.,* 2016). Indeed, different fat mass distribution exerts distinct effects on metabolic physiology, as abdominal adiposity is highly correlated with metabolic syndrome disorders and increased mortality (Chamorro-Garcia & Blumberg, 2014; Grün & Blumberg, 2009).

For a standard length of zebrafish larvae between 7.5 and 9 mm, the main anatomic locations for WAT were situated from the anterior dorsal limits of the visceral cavity to the rectum, above and around the two swim bladder chambers, close to the first loop of the anterior intestine, the posterior intestine and the rectum (Tingaud-Sequeira *et al.,* 2011). In our study (total length of 6.55 ± 0.56 mm), we observed lipid accumulations in the same locations and we also noted that the appearance of WAT was mostly correlated to the size of the larvae rather than the age (data not shown) (Riu *et al.,* 2014; Tingaud-Sequeira *et al.,* 2011).

Since fat build-up is controlled by the balance between lipogenesis and lipolysis (Kersten, 2001), we have included in our study the assessment of the impact that exposure to contaminants can have on both lipogenesis and adipogenesis (PPARγ:RXRα heterodimer, SREBP1, FASn) and also on fatty acid oxidation (ACOX1). Bodies of zebrafish larvae obtained at the end of the *in vivo* assay were analyzed by quantitative real-time PCR to determine whether the lipid accumulation verified *in vivo* was accompanied by changes in the levels of mRNA of key transcription factors and enzymes ruling lipid homeostasis. The results have demonstrated a differential expression in all genes following exposure to at least one of the treatments, with the exception of ACOX1, the enzyme responsible for fatty acid catabolism through β-oxidation in peroxisomes (Lyssimachou *et al.,* 2015).

Fatty acid synthesis begins with the conversion of acetyl-CoA to malonyl-CoA, a process catalyzed by acetyl CoA carboxylase (ACC), followed by the synthesis of a palmitate, by the FAS enzyme from malonyl-CoA, acetyl-CoA and NADPH, which is subsequently esterified into a triglyceride (Capitão *et al.,* 2017; Guan *et al.,* 2016).

Surprisingly, even though the accumulation of lipids was evident for TBT treatments at both concentrations, no relevant changes in gene expression were detected at a molecular level. Only the mRNA for lipogenic enzyme FASn was increased compared to the controls, at the TBT concentration of 100 ng/L Sn (lowest concentration tested). These observations could be explained if a mechanism of negative feedback was occurring, set to restore the levels of lipids and mRNAs back to initial state. Regardless, as FASn is the enzyme responsible for *de novo* lipogenesis, the increase in its expression alone could indicate that lipids were still being produced in both TBT 100 ng/L Sn and WWTP treatments. Hence we hypothesize that these changes at a molecular level are indicative of posterior changes at a physiological level and that it could be the reason why in WWTP treatments the alterations in gene expression are more evident than in other treatments, while the lipid accumulation is less visible. It seems possible that if the time of exposure had been extended, the treatment with 1.25% of influent could have resulted in a significant increase in lipid

accumulation, as the impact of the influent at a molecular level in the larvae exposed to that concentration was already verified. Regarding the TBT treatments, we hypothesize that a mechanism of negative feedback could be in place, therefore diminishing the expression of the transcription factors and enzymes that would increase the lipid content.

Another interesting detail in FASn expression is that in both treatments (TBT and WWTP influent), the highest concentrations appear to lead to a decrease in its mRNA levels when compared to the lowest concentrations, suggesting an inverted U-shaped dose-response curve, even though the differences between both doses were not considered statistically significant. This type of nonlinear dose-response relationship is apparently common in endocrine studies, where the highest concentrations lead to down-regulation, suppression or even compensation of the initial responses, in a phenomenon called "hormesis" (Lyssimachou *et al.,* 2015). Regardless, for now we cannot affirm the existence of a dose-response curve, based on so few concentrations tested.

Other studies have reported increases in lipid accumulation that were not accompanied by changes in gene expression. Watt and Schlezinger (2015) reported that after treating bone marrow mesenchymal stem cells of male of 9-week-old C57BL/6J mice with rosiglitazone (100 nM), TBT (100 nM), TPhT (10, 50 and 80 nm), MEHP, METBP or TBBPA (10 and 20 nM), they observed lipid accumulation for all toxicants, yet TBT, TPhT and rosiglitazone had no statistical increases in PPARγ levels of mRNA, even though the downstream target genes had their expression up-regulated. In contrast, MEHP, TBBPA and METBP led to lipid accumulation and increase in levels of PPARγ and target genes, with METBP having, overall, the least potent action among these three chemicals (Watt & Schlezinger, 2015).

Lipid metabolism in vertebrates is regulated through three different ways: allosteric regulation which comprises the control of enzyme activities through the binding of an activator or an inhibitor, post-translational modifications (e.g. phosphorylation) that can activate or deactivate the enzymes and transcriptional regulation controlled by transcription factors (Capitão *et al.,* 2017; Wang *et al.,* 2015). Nuclear receptor's transcription is regulated by phosphorylation and dephosphorylation events mediated by specific kinases (Lyssimachou *et al.,* 2015).

The most well-known transcription factors regulating adipogenesis and lipogenesis, which have a broad overlap in their transcriptional targets, include the SREBPs, the CCAAT/enhancer binding proteins (C/EBPs) and the nuclear receptors that form heterodimers with RXR [PPARs, pregnane X receptor (PXR), liver X receptor (LXR) and farnesoid X receptor (FXR)] (Capitão *et al.,* 2017; Grün & Blumberg, 2007; Lyssimachou *et*

al., 2015; Moseti *et al.,* 2016). Many other factors, such as insulin, glucocorticoids and Insulin-like growth factors (IGFs), influence adipogenesis directly or indirectly by stimulating the expression of some of the previously mentioned genes (Lyssimachou *et al.,* 2015). C/EBPβ, SREBP1 and insulin stimulate the expression of PPARγ. SREBP-1, stimulated by insulin secretion, is involved in both cholesterol and fatty acid synthesis and regulates the expression of *de novo* lipogenesis enzymes FASn and ACCα (Capitão *et al.,* 2017; Kersten, 2001; Lyssimachou *et al.,* 2015). The RXRα:PPARγ heterodimer, alongside C/EBPα, regulates the expression of adipogenic and lipogenic genes. C/EBP-family members also regulate the expression of 11beta-hydroxysteroid dehydrogenase (11β-HSD) 1 and 2, responsible for glucocorticoid homeostasis, and IGF-I and II, which play roles in growth, cell proliferation and development (Kersten *et al.,* 2001; Lyssimachou *et al.,* 2015).

Alongside SREBP1, LXR is a key regulator of cholesterol metabolism, although its effects are opposite to the former, as it decreases the cellular cholesterol levels (Hölttä-Vuori *et al.,* 2010). The SREBP family are membrane-bound transcription factors which directly regulate the expression of more than 30 genes involved in synthesis and uptake of cholesterol, fatty acids, triglycerides and phospholipids (Horton *et al.,* 2002). Just like PPARγ, SREBPs are also subjected to PTMs, such as phosphorylation, acetylation, ubiquitination and sumoylation, which can affect the processing, transcriptional activity and stability or degradation of the proteins (Wang *et al.,* 2013).

In our study, the expression of transcription factors RXRα, PPARγ and SREBP-1 was influenced by the environmental mixtures. Curiously, while RXRα and SREBP-1 were significantly overexpressed at both concentrations of WWTP influent, the PPARγ levels were clearly repressed at the lowest concentration of 1.25% and were not significantly affected at the concentration of 2.5%.

Many studies report the deregulation of lipid homeostasis *in vivo*, following TBT exposure, associated with transcriptional alteration of lipogenic and adipogenic genes. Lyssimachou *et al.* (2015), exposed *Danio rerio,* from pre-hatch to 9 months, to lower environmental concentrations of TBT (10 and 50 ng/L Sn) than the ones used in our study and reported changes in body weight, condition factor, hepatosomatic index, hepatic triglycerides and also changes in the expression of key transcription factors and enzymes involved in adipogenesis and lipogenesis [RXRα/a, PPARγ, SREBP1, FASn, ACOX1, C/EBPα, C/EBPβ, carbohydrate-responsive element-binding protein (ChREBP), acetyl-CoA carboxylase alpha (ACCα), diacylglycerol O-acyltransferase 2 (DGAT2)], glucocorticoid metabolism (11β-HSD2 and 11β-HSD3α) and growth and development (IGF-I and IGF-IIα) in the brain and liver of the fish. They have also identified the brain as a novel

target for TBT action and observed a sexual dimorphism in both morphological parameters (e.g. body weight, condition factor, liver triglyceride content and hepatosomatic index) and transcription of genes in the liver, as well as a tissue-specific impact of TBT in the metabolism.

In comparison to our study, Lyssimachou *et al.* reported an up-regulation of the expression of PPARγ in the liver in both genders (while the brain levels remained unchanged), with a concomitant increase in hepatic triglyceride levels, upregulation of SREBP1 and its encoding enzyme FASn in males. The transcription of RXRα in the liver was down-regulated in males at 50 ng/L Sn and upregulated in females at 10 ng/L, while in the brain a down-regulation was observed for both genders at the lowest concentration tested. In the brain of males, FASn was downregulated at 10 ng/L, while SREBP1 was significantly upregulated at 50 ng/L along with the FASn levels, although not statistically significant. In the brain of females, ACOX1 levels were significantly decreased. Similarly to our study, statistically altered gene expression was not always paired with lipid accumulation. The female fish at the lowest TBT concentration (10 ng/L) demonstrated an upregulation of the expression of RXRα and PPARγ, among other lipogenic/adipogenic genes, which was not coupled with an increase in hepatic lipid accumulation, and male fish exposed to 50 ng/L showed an upregulation of *de novo* lipogenesis genes SREBP1 and FASn, although no significant increases in triglyceride levels were observed.

Moreover, 11β-HSD2, the enzyme that deactivates cortisol was down-regulated in the liver and up-regulated in the brain, while 11β-HSD3α, the cortisol synthesizing enzyme, was down-regulated in the brain, which is consistent with previous reports indicating that TBT might interfere in glucocorticoid homeostasis (Grün & Blumberg, 2009).

Likewise, the effects of TBT on lipid homeostasis in other teleosts species (*Oncorhynchus tshawytscha*, *Carassius auratus* and *Sebatiscus marmoratus),* with observed effects such as increases in lipid content, number and size of adipocytes (adiposity), altered body weight and expression of genes in the downstream pathways of nuclear receptors such as PPARγ and RXR were reported (Capitão *et al.,* 2017).

PPARγ also plays an important role in lipogenesis in organs where it is minimally expressed, as it is the case of the liver, where the increase in expression is linked to hepatic triglyceride accumulation (Kersten, 2001). Nonetheless, in hepatocytes, the main mediator of the expression of lipogenic genes is SREBP-1, in contrast to the adipose tissue, where PPARγ has the most important role (Kersten, 2001). Since these genes, among others, are mostly expressed in specific organs, the whole-body evaluation of their levels of mRNA, as we applied in our study, might not have been the best strategy to detect statistically

significant differences in their expression after chemical exposure. The small size of our larvae did not allow an organ-specific assessment of the mRNA levels, hence the obtained information might not accurately reflect the changes in gene expression that might have happened in certain organs, leading to a sub-estimation of the differences detected between the control and contaminated treatments, especially for TBT treatments.

Other compounds have been shown to influence lipid homeostasis *in vivo* and *in vitro*, with changes in the expression of key genes.

Perfluorononanoic acid (PFNA), a perfluoroalkyl acid with wide commercial and consumer applications that can be found in environmental matrices, has been shown to decrease body weight, body length and hepatosomatic index of both zebrafish sexes, after exposure of 5-month old zebrafish for 180 days at concentrations of 0.01 (environmentally relevant), 0.1, and 1.0 mg/L in water. In contrast, the liver cholesterol levels increased in both sexes, whereas the liver triglyceride content was increased in males and decreased in females, matching the observation that the PFNA accumulates more in male livers than in females'. Moreover, PPARs and C/EBPs gene expression was enhanced in males and depressed in females (Zhang *et al.,* 2012). The authors suggested that the decrease in fatty-acid binding proteins (FABPs), PPARs, C/EBPs and triglyceride content in female livers could be due to a liver-ovary feedback loop, as female ovaries require a substantial amount of lipids during their development, thus promoting a liver-mediated diversion of the lipids towards the ovaries.

Exposure of BALB/c mice for 14 days to PFNA also led to a decrease in body weight (at 3 and 5 mg/Kg PFNA per day via gastric gavage), upregulation of the expression of PPARα and γ in the thymus (at 1 mg/kg PFNA per day) and increase in cortisol and adrenocorticotrophic hormone levels in sera, although no changes in the expression of the glucocorticoid receptor in the thymus were detected (Fang *et al.,* 2008).

The effects of MEHP (a metabolite of DEHP) and rosiglitazone on gene expression were investigated using a collection of 17 000 mouse cDNAs in gene expression array analyses and the results indicated that the majority of the genes were regulated by both chemicals, suggesting that the effects of MEHP are possibly mediated by PPARγ as well. Both catabolic and anabolic pathways were affected, with up-regulated genes belonging to the β-oxidation, citrate cycle, glucose uptake and glycolysis, oxidative phosphorylation and genes required for lipid droplets' structure and function of mature adipocytes (lipogenesis, triglyceride synthesis and adipokines). However, rosiglitazone specifically regulated around 30% of the total analyzed genes, showing a broader action than MEHP (Feige *et al.,* 2007).

Chamorro-Garcia and colleagues tested the obesogenic effects of TBT and rosiglitazone on C57BL/6J mice at a transgenerational level. After developing an assay where the parental generation (F0) was exposed through drinking water to rosiglitazone (500 nM) and environmentally relevant concentrations of TBT (5.42, 54.2 and 542 nM), the F1 generation was exposed *in utero*, the F2 exposure could only result from exposed germ cells and F3 generation had no exposure at all, they have noted that TBT led to an increase in WAT, increase in hepatic lipid accumulation, alteration in the expression of genes related to lipid storage and transport, lipogenesis as well as lipolysis, and biased MSC programming towards the adipocyte lineage at the expense of osteogenic lineage in all generations. Among the analyzed target genes, the ones mentioned in our study, PPARγ, PPARα, SREBP-1, FASn and ACOX, were all up-regulated in both males and females of all three generations exposed to either TBT or rosiglitazone. Similarly to our study, the main WAT depots that suffered increases after TBT-exposure were located in the visceral area especially in F2 males, and to a lesser extent in F1 males. Other locations for increases in WAT included the perirenal and interscapular depots. The F3 males showed significant increases in the adipocyte sizes and number and in the weights of all WAT depots, confirming the presence of transgenerational effects. Within the animals exposed to rosiglitazone, the only statistically significant differences in WAT were observed for F1 males, with increases in the epididymal depot weight and adipocyte size. Once again, sex-specific effects were noted, with the females showing more modest changes in adipose tissue and genes expression profiles even after TBT exposure (Chamorro-Garcia *et al.,* 2013).

In our study, a gender-specific evaluation of the effects was not possible to be obtained, as the zebrafish on the last day of the assay (18th-day postfertilization) had not yet attained sexual differentiation, which usually begins around the 25th day postfertilization (Santos *et al.,* 2017).

The effects of BPA and analogs have been observed in other teleost species. Juvenile sea bream fish (*Sparus aurata*) contaminated through the diet for 21 days with xenoestrogens BPA, nonylphenol and octylphenol (all with two doses: 5 mg/kg and 50 mg/Kg of body weight), showed significant increases in hepatic lipid accumulation and steatosis and overexpression of nuclear receptors involved in lipid homeostasis PPARα, PPARβ and PPARγ at the highest doses, lower doses and all doses of all chemicals, respectively. RXR and FAS levels were also significantly increased by nonylphenol, respectively, at the highest dose and all doses. The lowest concentration of BPA also upregulated RXR and at the highest concentration, an increase in FAS mRNA levels was observed. Moreover, these contaminants also led to a reduction in food intake after

exposure to all chemicals, although an increase in plasma cortisol levels was detected in nonylphenol- and octylphenol-exposed fish (Maradonna *et al.,* 2015).

Similarly, five-month old rare minnow *Gobiocypris rarus,* exposed to waterborne BPA at concentrations of 15 µg/L for 28 days, exhibited a significant decrease in serum triglyceride content of females, a non-significant increase in hepatic and serum triglyceride content in males alongside an upregulation of lipid synthesis demonstrated by the increased activities of *de novo* lipogenesis enzymes ACC and FASn in both sexes and glycerol-3-phosphate acyltransferase (GPAT) in males, even though fatty acid β-oxidation was also enhanced [increased activity of carnitine palmitoyltransferase I (CPT1), considered the main regulatory enzyme of long-chain fatty acid oxidation, in the liver of males]. Clear gender-specific effects were observed, as females displayed decreased GPAT and CPT1 activity, which was consistent with the decrease in triglyceride content (Guan *et al.,* 2016). Another particularly interesting detail in this study is that the changes in gene expression were not always consistent with the respective enzyme activity (e.g. reduced mRNA levels of GPAT but increased activity of the enzyme in males), which could possibly occur due to a negative feedback regulation. Regardless, the common assumption that mRNA expression has a direct correspondence with protein expression (central dogma of molecular biology) or protein activity is not fully correct, as many factors influence the correlation between them, such as different half-lives and post-transcription machinery (Benninghoff, 2007; Haider & Pal, 2013). The advantage of studying proteins instead of transcripts lies on the fact that they are truly responsible for the phenotypes of cells, organs and organisms and for the cellular responses to physiological stimuli, while mRNA is only the first step of a long sequence of events culminating in protein synthesis (Benninghoff, 2007; Graves & Haystead, 2002).

To further understand the molecular mode of action of the environmental samples and TBT, we have performed transactivation assays with the human and zebrafish PPARγ and RXRα receptors, as well as with the RXRα:PPARγ heterodimer.

Nuclear receptors' ligands are usually small hydrophobic molecules (Castro and Santos, 2014; Zhao *et al.,* 2015), yet very diverse and since PPARs are known to have a large ligand-binding pocket, it is no surprise that many different groups of chemicals have been shown to interact with these nuclear receptors (Capitão *et al.,* 2017).

Our results indicate that both the WWTP influent and the TBT are able to interact with the receptors *in vitro*, which might possibly be one of the mechanisms underlying the observed lipid accumulation *in vivo*. TBT at a concentration of 250 nM transactivated the *Homo sapiens* RXRα and heterodimer, but did not significantly transactivate PPARγ, even

though there appears to be an increase in activity in the *Homo sapiens* PPARγ. Surprisingly, the results for the *Danio rerio* receptors show a different tendency when compared to the human receptors. While the zebrafish RXRα activity was significantly induced and the PPARγ did not appear to be influenced by TBT, the heterodimer was massively repressed at the concentration tested. Our transactivation results for the zebrafish PPARγ and heterodimer seem to be in accordance with the results obtained for the expression levels of PPAR in our *in vivo* assay. Indeed, although an increase in PPARγ expression would be expected to lead to an increase in lipogenesis, what we have observed is that the statistically significant accumulation of fat in both TBT and influent treatments was either accompanied by no changes in PPARγ expression (TBT treatments and 2.5% influent) or even a decrease in its expression (at 1.25% influent).

The mammalian nuclear receptors RXRα and PPARγ possess orthologues in fish, which are expressed since early development (Lyssimachou *et al.,* 2015). The three PPAR isoforms (α, β and γ), which are critical lipid sensors and regulators of lipid metabolism, are conserved in the zebrafish and have similar distribution and roles as they have in mammals (Hölttä-Vuori *et al.,* 2010), even though teleosts possess five PPARs (Capitão *et al.,* 2017; Santos *et al.,* 2012). Five of the RXR sequences found in the zebrafish were analyzed and both their DNA-binding domain (DBD) and ligand-binding domain (LBD) have high sequence similarities (from 88 to 99%) to those of humans, supporting the idea of similar ligand affinities (Ouadah-Boussouf & Babin, 2016). More specifically, both DBD and LBD of RXRα from *Danio rerio* have a % of similarity with the human amino acid sequences of 99%, while the PPARγ amino acid sequences are similar in only 94% at the DBD and 74% at the LBD (Zhao *et al.,* 2015). Hence, the zebrafish PPARγ binding specificity is possibly different from the human's.

The position 285 of *Danio rerio* PPARγ contains a tyrosine, oppositely to the *Homo sapiens* PPARγ which holds a cysteine in the homologous position (Capitão *et al.,* 2017). It is possible that the mutation existent in the zebrafish PPARγ is the reason behind the disparity between the transactivation results, as differences in the nuclear receptor's structures may lead to distinct chemical binding and activation outcomes, besides the fact that the cysteine in the human PPARγ was proven to be essential for the activation of this receptor by TBT, as this chemical did not bind to the receptor when the residue Cys285 of the LBD was mutated into a residue of alanine (Capitão *et al.,* 2017; Harada *et al.,* 2015). Indeed, the disadvantage of using zebrafish is that, due to the whole-genome duplication that occurred in teleost evolution, the species acquired two more pairs of chromosomes compared to the human being and thus, changes in the expression of these paralog genes are not easily extrapolated to other vertebrates (Hölttä-Vuori *et al.,* 2010; Hill *et al.,* 2005).

Surprisingly though, this mutation in zebrafish PPARγ is not present in other Actinopterygii members *Sparus aurata* and *Pleuronectes platessa*, which maintain the cysteine molecule in the correspondent position (Capitão *et al.,* 2017).

Although TBT is known for being an agonist of the PPARγ, and despite the label of this receptor as the "Master regulator of adipogenesis", it has been suggested that TBT might activate the heterodimer through RXR, since this chemical was also reported to interact with other permissive heterodimers (LXR:RXR heterodimer) (Capitão *et al.,* 2017; Le Maire *et al.,* 2009). Similarly, Le Maire *et al.,* (2009) confirmed the transactivation of RXR:PPARγ by TBT and reported the covalent binding of TBT to the Cys432 of human RXRα as a crucial event for the activation of this receptor. They have also suggested that the interaction of TBT with the Cys285 of PPARγ does not allow an efficient stabilization of the active receptor conformation, besides the fact that organotins have been shown to bind to the receptor via a non-covalent ionic bond between the tin atom and the cysteine (Harada *et al.,* 2015), thus explaining why the TBT-dependent modulation of the activity of the heterodimer is most likely to occur through the binding to RXR instead. Indeed, our results also show that TBT is a far more powerful agonist of RXRα in both species, when comparing to PPARγ.

Moreover, Ouadah-Boussouf and Babin (2016) demonstrated that the obesogenic effect of TBT on the zebrafish RXR:PPARγ was not inhibited by the human PPARγ antagonist T0070907, but was fully abolished by the human RXR antagonist UVI3003 and by a combination of human RXR:RXR and RXR:PPARγ antagonists, once again proving that RXR plays a central role in TBT-modulated obesogenic outcomes and that *in vivo* obesogenic effects might occur through RXR-dependent pathways which do not necessarily involve PPAR isoforms.

Indeed, our study was not the only one showing a lack of transactivation of zebrafish PPARγ by TBT *in vitro* (Riu *et al.,* 2014).

Several other compounds have been shown to activate nuclear receptors involved in lipid homeostasis *in vitro*.

Bisphenol A and TBBPA are manufactured chemicals that can originate a variety of analogs when reaching the environment. TBBPA is debrominated into monoBBPA, diBBPA and triBBPA. TCBPA, although also produced for its use as flame retardant like TBBPA, can also be formed spontaneously in the environment from chlorination of BPA. The halogenated BPA derivatives (TBBPA and TCBPA) have been shown to activate human ERα, ERβ, and PPARγ (Riu *et al.,* 2011).

Despite being a model xenoestrogen, BPA was shown to have a much lower estrogenic activity (ability to bind to estrogen receptors α and β) than 17β-estradiol and chlorinated BPA analogs, while the brominated analogs are less estrogenic than BPA, with their potency decreasing as the number of bromine atoms increases. The transactivation of ERα and ERβ by these compounds followed the ranking order BPA > monoBBPA > diBBPA > triBBPA with TBBPA showing no estrogenic activity at all, whereas in the assays with PPARγ, the compounds were ranked in the inverse order (triBBPA = TBBPA > diBBPA > monoBBPA), with BPA showing no influence on PPARγ activity (Riu *et al.,* 2011).

TBBPA and TCBPA are agonists of human PPARγ, although their activity is about 100-fold less potent than rosiglitazone. Furthermore, they have triggered a similar PPARγ activation at 10-to 100-fold lower concentrations (micromolar range) than known PPARγ ligands MEHP, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), and when in the presence of [^{3}H]-rosiglitazone, these compounds competitively inhibited the binding of the later to the receptor. Rosiglitazone's larger size and higher number of hydrogen bonds formed with the ligand binding protein of the PPARγ, when compared to BPA and halogenated BPAs, could explain its higher affinity for the receptor. TBBPA, TCBPA, MEHP (all at 10 μM) and rosiglitazone (at 1μM) were tested for their ability to bind to human, zebrafish and *Xenopus* PPARγ receptors. Among these, TBBPA, TCBPA and MEHP all activated the human and zebrafish PPARγ, with MEHP being the weakest ligand of the zebrafish receptor while rosiglitazone did not activate the zebrafish receptor, and all compounds activated the *Xenopus* PPARγ. The authors also suggested that the replacement of human PPARγ Gly284 by serine residues and Cys285 by tyrosine residues in the zebrafish receptor could be the reason behind the observed reduced binding affinity of rosiglitazone, in the same way that the different binding mode of the halogenated BPAs would allow their binding to both human and zebrafish PPARγ (Riu *et al.,* 2011).

To further investigate the ability of these compounds to induce adipogenesis, Riu and collaborators analyzed the adipocyte differentiation (using NIH3T3L1 preadipocytes) after exposure through Oil Red O staining and verified that TCBPA and TBBPA at 10 μM induced adipogenesis, while co-treatment with PPARγ antagonist CD5477 inhibited the adipocyte differentiation by TBBPA, suggesting that this chemical mediates its effects on adipogenesis through PPARγ. Furthermore, TBBPA and TCBPA led to a similar expression of PPARγ gene and diminished expression of its target gene ApoA2/FABP4 (AP2) when compared to the treatment with rosiglitazone (Riu *et al.,* 2011).

The potential of halogenated-BPAs as human PPARγ ligands has also been verified *in vivo*, using a transgenic line of zebrafish embryos [*Tg*(*h*PPARγ-*e*GFP)] to investigate

whether the receptor was activated and where this activation occurred in the whole organism (Riu *et al.,* 2014). A 24-hour exposure to rosiglitazone led to a strong green fluorescent protein (GFP) expression in the epidermis of the whole embryo, while exposure to 100 nM and 1 µM TBBPA and 1 µM TCBPA, led to the expression of GFP to a lesser extent, focused on the head and tail regions. TBBPA-S (1 µM), a sulfate-conjugated metabolite of TBBPA formed in the larvae, also activated the receptor *in vivo*. TBBPA, TCBPA and TBBPA-S exhibited a weak agonistic activity towards zfPPARγ *in vitro*, with TBBPA being the most potent, whereas TZDs (rosiglitazone, pioglitazone, ciglitazone and troziglitazone), a prostaglandin and TBT did not activate the receptor *in vitro* (Riu *et al.,* 2014). The authors have concluded that known human PPARγ ligands can therefore not be used as positive controls in assays involving the zebrafish PPARγ. Indeed, this conclusion matches the results obtained in our study, as the well-known obesogen TBT did not influence PPARγ transactivation in the zebrafish.

Feige *et al.,* (2007) showed that different PPARγ ligands (DEHP and rosiglitazone) lead to the recruitment of distinct coactivators and selectively regulate the interactions between coregulators, which not only has an impact on the size of the transcriptional complex, but can also influence the different activities and intensities of the physiological effects noted between ligands, since it might lead to a differential gene regulation.

Feige and colleagues (2007) described that the obesogenic effects of MEHP on 3T3L1 cells were lower than those induced by rosiglitazone, yet the effects of this chemical were also shown to require the PPARγ, as the treatment with the GW9662 antagonist and PPARγ knock-down significantly inhibited the adipogenic action of both MEHP and rosiglitazone treatments. MEHP was shown to have a similar binding mode to the PPARγ LBD when compared to rosiglitazone (same residues contacted: Ser289, His323, His367 and Tyr473), yet as MEHP promotes interactions with only a subset of PPARγ coregulators, the conformational changes provoked by these ligands on the receptor are different. Furthermore, MEHP activated mouse PPARα, β and γ and human PPARγ, but with a maximal activation at 100 µM of about half of the achieved with rosiglitazone. Regardless, the effects were noted at levels of 3.2 µM, which is very concerning, since plasmatic levels of DEHP and MEHP as high as 50 µM have been reported in humans (Feige *et al.,* 2007).

Takacs and Abbott (2007) evaluated the ability of PFOA and PFOS to activate mouse and human PPARα, β and γ, at concentrations within the ranges found in rats and mice maternal serum (0.5-250 µg/mL), using Cos-1 cells in transient transfection assay following a 24h period of exposure. PFOA's transactivation potential was higher than PFOS in both species, with PPARα and β activities being enhanced, while PPARγ activation was not

significant for either of these chemicals. Moreover, the PPARα antagonist MK-886 suppressed the effects of both chemicals in both species, whereas the PPARγ antagonist GW9662 only suppressed the activation of the human isoform for PFOA. Troglitazone, used as a positive control, increased activity of both human and mouse PPARγ (Takacs & Abbott, 2007).

Both studies of Takacs and Abbott (2007), as well as Feige *et al.,* (2007), have pointed out the existence of a disparity in results as different cell types were used. Particularly, Feige *et al.* reported different efficacies of MEHP (relative to the reference agonist rosiglitazone) to transactivate PPARγ in different cell lines, reaching 80% of the reference activation level in C2C12 cells, 60% in COS-7 cells and only 35% in HeLa cells (Feige *et al.,* 2007). These observations were even more accentuated when Takacs and Abbott compared their lack of PPARγ activity after PFOA exposure on COS-1 cells, to the results reported by Vanden Heuvel *et al.,* where this chemical activated PPARγ in 3T3-L1 cells at the same concentration tested (100 µM) (Takacs & Abbott, 2007; Vanden Heuvel *et al.,* 2006). Thus, the ability of certain compounds to activate nuclear receptors might be cell-type dependent.

Fibrates are examples of pharmaceuticals, used to treat hypercholesterolemia in humans, which are ubiquitously present in aquatic ecosystems and lead mostly to a depletion of lipid levels (Capitão *et al.,* 2017; Coimbra *et al.,* 2015). A multigenerational study on zebrafish with a whole life-cycle exposure of an F0 generation to the clofibric acid, a metabolite of the pharmaceutical clofibrate designed to lower blood lipid content through its action of PPARα, at concentrations of 1 and 10 mg/g (diet), reported differential effects across generations. The F0 fishes had a significant reduction in weight and muscle triglyceride levels parallel with increases in liver mRNA levels of PPARα and ACOX1, oppositely to their offspring (F1 generation) which exhibited an increase in weight with a concomitant upregulation of PPARγ and down-regulation of PPARβ, involved in lipid mobilization, at the highest concentration tested (Coimbra *et al.,* 2015).

The activation of PPARα by fibrates is well described in the literature. Ciprofibrate has been used as a positive control for testing of human and mouse PPARα activation by new environmental chemicals (Vanden Heuvel *et al.,* 2006) and as previously mentioned, fenofibrate is also a PPARα agonist, besides its interference in glucocorticoid homeostasis (Fang *et al.,* 2008).

In addition to the already mentioned alterations of PPARs gene expression, the alkylphenols nonylphenol and octylphenol have also been reported to activate the human estrogen receptor, a possible mode of action of many obesogens (Bonefeld-Jørgensen *et al.*, 2007). Bonefeld-Jørgensen and colleagues reported that BPA, BPA dimethacrylate,

nonylphenol and octylphenol all exhibited estrogenicity (BPA and nonylphenol having the highest potency compared with 17β-estradiol), antagonized the androgen receptor transactivation (in a similar order of potency), inhibited the aromatase activity and interacted with the aryl hydrocarbon receptor (involved in steroid synthesis and metabolism) *in vitro*.

Rosiglitazone, MEHP, METBP, TBBPA and the organotins TBT and TPhT were all shown to activate the mouse PPARγ1 and 2 in Cos-7 cells, with the TZD and the organotins having the highest potencies and efficacies, whereas the remaining chemicals only acted as partial agonists (Watt & Schlezinger, 2015).

Regarding our transactivation results with the influents, only the concentration of 10% pure influent caused a statistically significant effect in the transactivation of the human heterodimer, even though there appears to be a dose-dependent increase in the activity of both human and zebrafish heterodimers. It is important to take into consideration that the concentrations of 1.25% and 2.5% tested correspond to the concentrations used in our *in vivo* assay, where zebrafish larvae were chronically exposed from the 4th to the 18th day postfertilization. Hence, the results are not easily comparable, since the transactivation assays include a short window of exposure of only 24 hours, which can explain the lack of transactivation at these low concentrations.

In relation to the WWTP influent fractions, no statistical results were obtained for any of the assays. Even though the concentrations of the fractions tested correspond to 20% of the pure WWTP influent, the total compounds are being divided into the 4 fractions (A, B, C and D) corresponding to different elution conditions, hence the transactivation caused by the fractions individually could be far less powerful than the transactivation obtained from pure influent where all the compounds are combined, as the simultaneous exposure to this "cocktail" of obesogens can potentially lead to additive, or even synergistic effects (e.g. by acting on both RXR and PPARγ simultaneously) (Biemann *et al.,* 2014; Riu *et al.,* 2011). On the other hand, as several compounds have also been shown to repress the activity of the nuclear receptors, the possibility that antagonistic actions of different chemicals on the nuclear receptors might exist cannot be rejected (Biemann *et al.,* 2014).

In general, our study has shown that complex environmental mixtures are able to deregulate metabolic pathways, leading to an increase in lipid accumulation and adipogenesis. So far, not many studies have reported the impact of chemical mixtures on obesity and related-disorders, which is concerning, as real-life exposure occurs to mixtures rather than to single chemicals. The effects of exposure to the environment are even less known, thus, we have included WWTP influents in both transactivation assays and the *in vivo* assay with zebrafish. WWTP influents contain a variety of potential obesogens,

alongside with other chemicals with a possible anti-obesogenic activity (e.g. fibrates, phytoestrogens and other estrogenic compounds). All the previously cited obesogens and anti-obesogens are routinely detected in the environment and WWTPs, alongside with many other EDCs that can possibly interfere with lipid homeostasis (Betts, 2007; Clara *et al.,* 2010; Huang *et al.,* 2012; Loos *et al.,* 2013; Martí *et al.,* 2011; Riu *et al.,* 2011). Moreover, WWTP samples are well-known for their estrogenicity (Loos *et al.,* 2013) and compounds with estrogenic activities, such as BPA and DES, have been linked to the disruption of lipid homeostasis (e.g. through activation of ERs) in a diversity of animal groups (Capitão *et al.,* 2017; Newbold *et al.,* 2009).

Possible mechanisms of obesogen action include the direct binding to nuclear receptors (such as the oestrogen receptor α and the PPARγ) by acting as either agonist or antagonist, and indirectly through the inhibition of enzymatic activity or activation of the expression of p450 enzymes (Bašić *et al.,* 2012). To further complicate the picture, the same compound can have multiple modes of action, as it is the case of TBT, that is also known to affect glucocorticoid homeostasis and the expression of aromatase mRNA and its enzymatic activity, as well as phthalates, known to activate PPARα while their metabolites have a preference for PPARγ, favoring a lipolysis state (Grün & Blumberg, 2009). Halogenated BPA derivatives (TBBPA and TCBPA) are other examples of chemicals with many possible mechanisms of action in the disruption of lipid homeostasis, by disrupting thyroid hormone receptor, estrogen receptors and the PPARγ signaling (Bašić *et al.,* 2012; Riu *et al.,* 2011) Moreover, besides having multiple modes of action, obesogens may also elicit compensatory mechanisms (Bašić *et al.,* 2012; Grün & Blumberg, 2009), which complicate the interpretation of results. Additionally, as lipogenesis and adipogenesis are commanded by a complex network of transcription factors and enzymes, all mechanisms could also interact with each other, leading to disruptions at many levels (Bašić *et al.,* 2012).

Being a permissive heterodimer, the RXRα:PPARγ can be activated by the binding of an agonist to either nuclear receptor (Grün and Blumberg, 2007). Hence, the outcomes of this chemical-mixture exposure could not be easily predicted. A future chemical characterization of our WWTP samples and fractions will allow a better understanding of what we are dealing with, as for now, we do not possess any information on the compounds and respective concentrations in the samples. Moreover, this step might eventually help us discover new potential obesogens.

Several authors have also pointed out the need to investigate the effects of mixtures of chemical obesogens to which the human being is exposed and understand their mode of action (Benninghoff, 2007; Biemann *et al.,* 2014; Chamorro-Garcia & Blumberg, 2014).

Moreover, many studies have mentioned the need to develop high-throughput large-scale screening techniques that could be used to evaluate the toxicity of many chemicals at once, in addition to single-chemical investigations (Benninghoff, 2007; Hill, 2005).

In that matter, Biemann *et al.* (2014) exposed multipotent murine mesenchymal stem cells (C3H10T1/2) to a mixture of BPA, DEHP and TBT, and concluded that the impact of this mixture on adipogenesis was not predictable from the effects of each compound individually. Whereas single BPA exposure decreased the number of adipocytes, triglyceride content and expression of adipogenic genes, and single DEHP and TBT massively induced the opposite effects, the mixture promoted adipogenesis although with a far less pronounced effects than the ones obtained with the single chemicals, as the TBT and DEHP effects overcame the single effects of BPA. Moreover, only the mix with high non-environmentally-relevant concentrations (10x the environmentally-leveled mix of 10 nmol/L BPA, 100 nmol/L DEHP and 1 nmol/L TBT) was able to result in an increase of adipogenesis and induce the expression of adipogenic marker genes. In either the mix with the highest concentrations, nor with the lowest concentrations, the triglyceride levels were changed, which was not expected considering the individual substances' responses and the fact that adipogenesis was clearly enhanced at the highest concentrations tested.

Lyche and collaborators (2013) exposed, through the diet, a parent generation (F0) of zebrafish to natural mixtures of persistent organic pollutants (POPs) extracted from the liver of burbot (*Lota lota*) from Lake Mjøsa and Lake Losna in Norway. The chemical analysis indicated the dominance of polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) metabolites in the Mjøsa Lake, whereas Lake Losna contained the same compounds yet with low levels of PBDEs. After performing a genome wide transcriptome profiling of the zebrafish 3rd-generation (F2) embryos and functional gene network analysis, they have noted that among the observed effects, carbohydrate and lipid metabolism were influenced by the exposure and identified the hepatocyte nuclear factor 4 alpha (HNF4A) as central regulators of the network (Lyche *et al.,* 2013). The same environmental samples, which were chemically quantified and the concentrations of POPs fit the levels reported in human and wildlife populations, resulted in increased weight of the exposed zebrafish (from 6 dpf to 5 months) at the end of the exposure period, and central positions of the network obtained through microarrays were occupied by key regulators of weight (PPARs, glucocorticoids, C/EBPs, estradiol), steroid hormone functions (glucocorticoids and estradiol) and insulin signaling (HNF4A, C/EBPs and PPARγ) (Lyche *et al.,* 2011).

In a study of exposure in the field, the Atlantic bluefin tuna (*Thunnus thynnus*) caught in the Strait of Messina in the central Mediterranean Sea, exhibited accumulation of PCBs (mostly dioxin-like PCBs) and organochlorine pesticides (OCPs) in the liver, more accentuated in males, concomitantly with an ectopic lipid accumulation in both sexes and high expression of PPARγ and RXRα genes (especially in males). Furthermore, a metabolomics approach indicated a significant hepatic glucose depletion from males to females, alongside an increase in levels of malonate, which indicate the occurrence of fatty acid biosynthesis in the liver. Once again, it was mentioned the possibility that the different accumulation pattern between sexes could be due to a lipid mobilization from the liver to the gonads to support egg development in females, and could also be attributed to less feeding during the reproductive period (Maisano *et al.,* 2016).

Sarotherodon melanotheron from a contaminated tropical freshwater dam (Awba Dam in Nigeria), rich in PCBs, OCPs, polycyclic aromatic hydrocarbons and heavy metals according to sediment samples chemical analysis, was compared to a reference site and it was described that the size of the contaminated fish was bigger and had higher levels of PPARα, β and γ mRNA levels in both sexes, although the PPAR protein levels were higher in the contaminated females in comparison to the contaminated males. The chemical concentrations found in the samples also correlated with the increase in the liver somatic index (Adeogun *et al.,* 2016).

In summary, it appears that the effects of some obesogens might be gender- and organ-dependent (e.g. BPA affecting mostly males and the liver) and might not affect the different species through the same regulatory pathways, since some nuclear receptors, which play central roles in the maintenance of lipid homeostasis, have been reported to have structural differences in their ligand binding domain which could lead to distinct ligand-binding affinities among species (e.g. TBT with human and zebrafish PPARγ). Unveiling the crystallographic structure of TBT binding to the human RXR and PPARγ has allowed a better understanding of the mode of action and interaction of this obesogen with the receptors. Similar studies should be performed with other chemicals and nuclear receptors to help elucidate the eventual different responses of species exposure to a particular obesogen.

As seen in our study, TBT does indeed increase *in vivo* lipid accumulation in *Danio rerio*, although no statistically significant differences in the studied transcripts compared to the control were detected and a significant repression of the RXR:PPARγ heterodimer *in vitro* was observed. Whether the zebrafish RXR:PPARγ regulates lipid homeostasis through its repression, instead of an activation as seen in the mammalian heterodimer, remains to be

investigated. Seeing that TBT activated the zebrafish RXR but repressed the heterodimer *in vitro*, it seems possible that other receptors forming heterodimers with RXR and that also play roles in the maintenance of the lipid homeostasis, such as the FXR and LXR, could be a more important vehicle for the observed effects of TBT *in vivo* in zebrafish, rather than the RXR:PPARγ heterodimer signaling pathway.

These receptors are also tightly related to some of the genes studied here. LXR is involved in cholesterol homeostasis and regulates the expression of SREBP-1, while PXR regulates lipid homeostasis and the expression of PPARγ in the liver (Capitão et al., 2017).

The interaction of chemicals with the RXR:PPARγ heterodimer is particularly concerning, not only as it may lead to lipid and glucose homeostasis perturbation but also due to the presence of PPARs in vertebrate and many invertebrate groups (Cephalochordates, Urochordates, molluscs and Echinoderms) and RXRs in most metazoans, which indicate that the taxonomic scope of this inappropriate regulation of the pathways is wider than initially expected (Lyssimachou *et al.,* 2015; Santos *et al.,* 2012). Moreover, RXR is the heterodimeric partner of several other nuclear receptor that are involved in lipid homeostasis to some extent [LXR (liver X receptor) and FXR (farnesoid X receptor) present in deuterostomes and the ecdysone receptor, a LXR/FXR orthologue in some protostomes], some of which also form permissive heterodimers (Capitão *et al.,* 2017).

Taking together our findings along with the ones in previous studies, compiled evidence points to an impact of the surrounding environment in lipid homeostasis, not only in human beings but throughout the animal kingdom. Indeed, more than 1300 chemicals have been identified as potential endocrine disrupting chemicals, yet only a few have been tested *in vivo*, and within this group, very little is known about their ability to disrupt lipid homeostasis (Capitão *et al.,* 2017). Being widely present in products of our daily life, with some examples including cosmetics, plastics, food cans and pesticide-treated food (Capitão *et al.,* 2017), obesogens threaten not only the human populations worldwide, but also the environment and ecosystems. In worst cases, these compounds persist in the environment either due to their physicochemical properties, which can also lead to their bioaccumulation and biomagnification, or for the simple fact that they are continuously being released into the environment (Capitão *et al.,* 2017).

Our study focused on the interaction of suspected obesogens with the pre-translational transcriptional regulation of the lipogenic and adipogenic genes and enzymes, yet obesogens have been shown to have several possible modes of action beyond the ones included here. Post-translational regulation of certain enzymes, such as CPT1 (Guan et al.,

2016) and ACC (Capitão et al., 2017; Wang et al., 2015) have been shown to modify their activity and affect the affinity for certain substrates (Guan et al., 2016), allowing a rapid regulation of lipogenic enzymes (Wang et al., 2015), therefore, it would be important to investigate whether obesogens and environmental samples are able to induce these modifications.

Since developing organisms, such as fetuses and neonates, are the most sensitive to chemicals with hormone-like activity (Newbold *et al.,* 2007), studying the effects of their exposure to these chemicals should be a priority. This particular sensitivity is due to the lack of fully functional protective mechanisms such as DNA reparations, an efficient immune system, good liver metabolism, detoxifying enzymes and a developed blood/brain barrier, therefore allowing toxic effects to occur at much lower concentrations of the chemical that the ones affecting the adult (Newbold *et al.,* 2007). In some cases, it is possible that effects revealed in offspring are not detected in the exposed progenitors (Newbold *et al.,* 2007), for example, obesogens affecting adipogenesis *per se* probably exert a higher impact during periods of adipose tissue differentiation (i.e. fetal development, perinatal nursing and adolescence) (Grün & Blumberg, 2009). Moreover, effects resulting from perinatal exposure may not manifest until later in life (e.g. adulthood) (Newbold *et al.,* 2007), thus, it is important to examine the consequences of exposure to obesogens in developing organisms, both during the development and after reaching the adult stage, but also at a transgenerational level.

So far, a few chemicals have been reported to cause obesity-related transgenerational effects in rats, such as plastic components (BPA, DEHP and dibutyl phthalate), a mixture of hydrocarbons, as well as DDT and possibly its primary metabolite dichlorodiphenyldichloroethylene (DDE, an anti-androgen) that has also been reported to lead to epigenetic modifications in sperm (Chamorro-Garcia & Blumberg, 2014).

The zebrafish short life cycle and rapid maturation facilitate the experimentation for transgenerational endpoints, such as the study of epigenetics (Hill *et al.,* 2005). Moreover, gene programming and development in early life stages (e.g. embryo) is conserved among vertebrates and transplacental transfer of chemicals to the offsprings in mammals is similar to the transmission of these chemicals from the female fish to the eggs (Hill *et al.,* 2005), making the zebrafish a suitable model for these studies.

Epigenetic alterations, such as DNA methylation, histone modifications or noncoding RNA, could be a potential target for obesogen action (Bašić *et al.,* 2012; Biemann *et al.,* 2014), hence whether obesogens promote transgenerational obesogenic effects through interferences with epigenetic mechanisms should be an object of future research.

In this regard, Manikkam and collaborators have exposed gestating Sprague Dawley female rats (generation F0) intraperitoneally and during embryonic days 8-14 of development, to a mixture of plastic derived compounds (BPA, DEHP and dibutyltin) and reported an epigenetic transgenerational inheritance of adult onset disease, from which obesity was one of the evaluated endpoints (Manikkam *et al.,* 2013). The F3 generation sperm epigenome revealed 197 differential DNA methylation regions in gene promoters (epimutations), some of them with a direct connection in the gene network associated with the identified pathologies, more specifically, 5 of these genes correlated to known obesity-related genes. Moreover, at the lowest doses (25 mg/kg BPA, 375 mg/kg DEHP and 33 mg/Kg dibutyltin per day), both F3 males and females suffered an increase in body weight, as well as abdominal fat deposition and adiposity in most organs.

Following a better understanding of the obesogens' role in obesity, focus should be applied on uncovering prime targets for pharmaceutical intervention, as the traditional dietary approaches fail to combat obesity. For this purpose, a complete picture of the network of interactions within the cell and between organs needs to be obtained. The advances of the *omics* such as genomics and proteomics have provided new tools for the discovery of biomarkers of both exposure and effects of the action of chemicals, as they apply high-throughput techniques that allow the visualization and comparison of the full expression patterns of cells or tissues, following exposure to chemicals, to a control state, and could help identify genes and proteins, regulated by each nuclear receptor, that are altered by obesogen exposure, like a "fingerprint" (Benninghoff, 2007; Capitão *et al.,* 2017). This "fingerprint" of exposure would aid in the prediction of the obesogen mode of action, although any sort of expression profile (gene and protein) is simply a snapshot of a highly dynamic system from which temporal changes can occur (Benninghoff, 2007). Proteomic approaches include two-dimensional gel electrophoresis (2DE) coupled with mass spectrometry (MS) for protein identification, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS), that could be used to identify differentially expressed proteins (Benninghoff, 2007).

Although PPAR activity could be pharmacologically controlled to treat metabolic disorders (Feige *et al.,* 2007), it is important to remember the existence of adverse effects of previous potent PPARγ agonists (e.g. TZDs). Hence, the discovery of selective modulators that could provide the beneficial effects of the current agonists, without their side effects, is one of the challenges in pharmaceutical research (Feige *et al.,* 2007).

To further elucidate the mechanisms of obesogen-mediated lipogenesis and adipogenesis, gene knockdown by morpholinos or other approaches such as CRISPR/Cas9 and transient or stable transgene expression assays could be performed, with zebrafish remaining a popular model as it is easily genetically manipulated (Hill *et al.,* 2005; Hölttä-Vuori *et al.,* 2010). Moreover, morpholinos or CRISPR/Cas9 targeting genes that are expressed differentially after exposure to an obesogen, could help prevent the toxicity of the latter (Hill *et al.,* 2005).

5. Conclusions

New compounds are emerging as ligands of nuclear receptors related to metabolic pathways of lipid metabolism, which supports the idea of a link between obesity and environmental contamination. The combination of *in vitro* assays focused on the activation of PPARγ and other receptors essential for lipid homeostasis and adipogenesis, along with *in vivo* assays, allows the first line screening of obesogens, followed by an examination and confirmation of the existence of obesogenic effects in animals. Additionally, bioinformatics help to integrate information for a better understanding of the obesogens' mode of action.

The exact role of environmental contamination in the development of obesity and related disorders is not yet fully uncovered. Indeed, the studies evidencing the additive or synergetic effects of both exposure to obesogens and feeding with high-fat diets (e.g. egg yolk) are many and some reported a lack of obesogenic effects when in the absence of this type of diet. These observations seem to suggest that in the absence of a high-caloric intake, the effects of obesogens are either very low or non-existent. Nonetheless, since the changes in dietary habits towards high caloric intake are an issue in most countries, the simultaneous assessment of the effects of obesogens and overnutrition might represent the current status of human life more accurately.

Furthermore, the many studies indicating the existence of a sexual dimorphism in the observed effects suggest that the genders should be evaluated separately. As different tissues and organs display distinct molecular responses following exposure to chemicals, organ-specific evaluations might be more informative than a whole-body assessment. Differences in the accumulation of chemicals between tissues might be the underlying cause for these differential responses.

As reviewed by Hölttä-Vuori *et al.,* (2010), the zebrafish is an attractive model in the study of lipid homeostasis, owning to a variety of advantages, among which the conservation of the mechanisms underlying lipid metabolism, between the zebrafish and mammals, play an important role. Hence, this species serves both for an assessment of the

effects of obesogens in the aquatic environment, as well as for the assessment of their effects on more complex animals, such as the humans. Regardless, we have concluded that the small differences among species can greatly influence the outcomes of exposure to obesogens, as it is the case of the structural differences between the LBDs of nuclear receptors which lead to distinct chemical binding specificities and affinities.

Overall, the present study shows that environmental samples and model obesogen TBT, at environmentally relevant concentrations, are able to increase the lipid accumulation and interfere with the transcriptional regulation of lipid homeostasis of zebrafish *in vivo*, by interacting with the PPARγ:RXR heterodimer or the RXR receptor and modulating the expression of key enzymes from downstream pathways of the adipogenic/lipogenic cascade.

Many of the presented studies, including ours, involved chemical concentrations found in the environment or human/animal blood and tissues. The most concerning cases are high levels of EDCs in either the cord blood or maternal milk, as they denote the presence of perinatal and early postnatal exposure within populations, which can be specifically hazardous during development, not only due to the lack of fully functional protective mechanisms, but also since some obesogens might bias undifferentiated cells towards the adipogenic lineage at the expense of other lineages (e.g. osteogenic). Therefore, studying the impact of early exposure in critical periods of the animal development and at a multigenerational and transgenerational level is essential to comprehend the true role of chemical exposure in the obesity epidemic.

Additionally, future studies should focus on the more realistic scenarios of exposure, which can be achieved through the evaluation of the outcomes of exposure to chemical mixtures and chemically-characterized environmental samples, since studies on single obesogens do not reflect the possible additional, synergistic or antagonistic relationships that can be observed between compounds in the environment.

The scientific community has become interested in the development of drugs that could decrease triglyceride synthesis and fat storage, in order to reduce adipose tissue mass and the transcription factors SREBP1 and PPARγ could indeed be attractive targets for pharmaceutical intervention, since they are differentially expressed after exposure to single obesogen chemicals and to mixtures. Our observations confirm that nuclear receptors could be targets of interest for the action of pharmaceuticals designed to treat obesity and related pathological conditions, however as these receptors take part in a variety of functions within the cells and tissues while commanding the expression of several genes, targeting them might lead to undesired outcomes, as it is the case with TZDs in diabetic patients. It is,

therefore, a priority to understand precisely what the consequences of targeting these receptors with new drugs are. Obtaining a complete picture of the network of interactions between genes or proteins within the organisms should be a major focus of the future research and could be facilitated through the application of the high-throughput *omic* techniques, which could potentially lead to the detection of a "fingerprint" of exposure and outcomes for obesogens.

Thus, for our future work, investigating new metabolic pathways, other transcriptional regulation mechanisms, other genes, enzymes and hormones involved in the maintenance of lipid homeostasis and their upstream regulators (e.g. nuclear receptors, such as PXR and LXR, and transcription factors), will help elucidate the underlying molecular mechanisms of chemical-induced obesity, which will be crucial for the development of pharmaceuticals and therapies capable of curbing obesity and associated pathologies. Lastly, a full characterization of our WWTP samples will allow a better interpretation of the obtained results.

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